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An ancient dental gene set governs development and continuous regeneration of teeth in sharks

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ABSTRACT

The evolution of oral teeth is considered a major contributor to the overall success of jawed vertebrates. This is especially apparent in cartilaginous fishes including sharks and rays, which develop elaborate arrays of highly specialized teeth, organized in rows and retain the capacity for life-long regeneration. Perpetual regeneration of oral teeth has been either lost or highly reduced in many other lineages including important developmental model species, so cartilaginous fishes are uniquely suited for deep comparative analyses of tooth development and regeneration. Additionally, sharks and rays can offer crucial insights into the characters of the dentition in the ancestor of all jawed vertebrates. Despite this, tooth development and regeneration in chondrichthyans is poorly understood and remains virtually uncharacterized from a developmental genetic standpoint. Using the emerging chondrichthyan model, the catshark (*Scyliorhinus* spp.), we characterized the expression of genes homologous to those known to be expressed during stages of early dental competence, tooth initiation, morphogenesis, and regeneration in bony vertebrates. We have found that expression patterns of several genes from Hh, Wnt/ β -catenin, Bmp and Fgf signalling pathways indicate deep conservation over ~450 million years of tooth development and regeneration. We describe how these genes participate in the initial emergence of the shark dentition and how they are redeployed during regeneration of successive tooth generations. We suggest that at the dawn of the vertebrate lineage, teeth (i) were most likely continuously regenerative structures, and (ii) utilised a core set of genes from members of key developmental signalling pathways that were instrumental in creating a dental legacy redeployed throughout vertebrate evolution. These data lay the foundation for further experimental investigations utilizing the unique regenerative capacity of chondrichthyan models to answer evolutionary, developmental, and regenerative biological questions that are impossible to explore in classical models.

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1. Introduction

There is currently a need to better understand the mechanism of tooth regeneration in an attempt to design more appropriate therapies targeted at human tooth loss (Jussila and Thesleff, 2012; Ohazama, 2005; Ohazama et al., 2004; Thesleff and Tummers, 2008; Zhang et al., 2005). Ultimately, the field of translational dental research aims to uncover what factors govern the transition from a single or restricted set of teeth to the development of multiple generations (Tucker and Fraser, 2014). Conversely, for a phylogenetic template, research should focus on the mechanisms associated with tooth generational loss for more restricted and

derived dentitions (Juuri et al., 2013). A deeper understanding of the extreme regenerative potential of diverse vertebrate dentitions may forward these goals. Mammals, e.g. mice, are currently well-known models for tooth development; however they have relatively limited regenerative capacity (Harada et al., 1999), and form complex tooth cusps within the primary teeth (Jernvall and Thesleff, 2012; Ohazama et al., 2010; Tucker and Sharpe, 2004), restricting their potential as complete models for odontogenesis, including complete replacement.

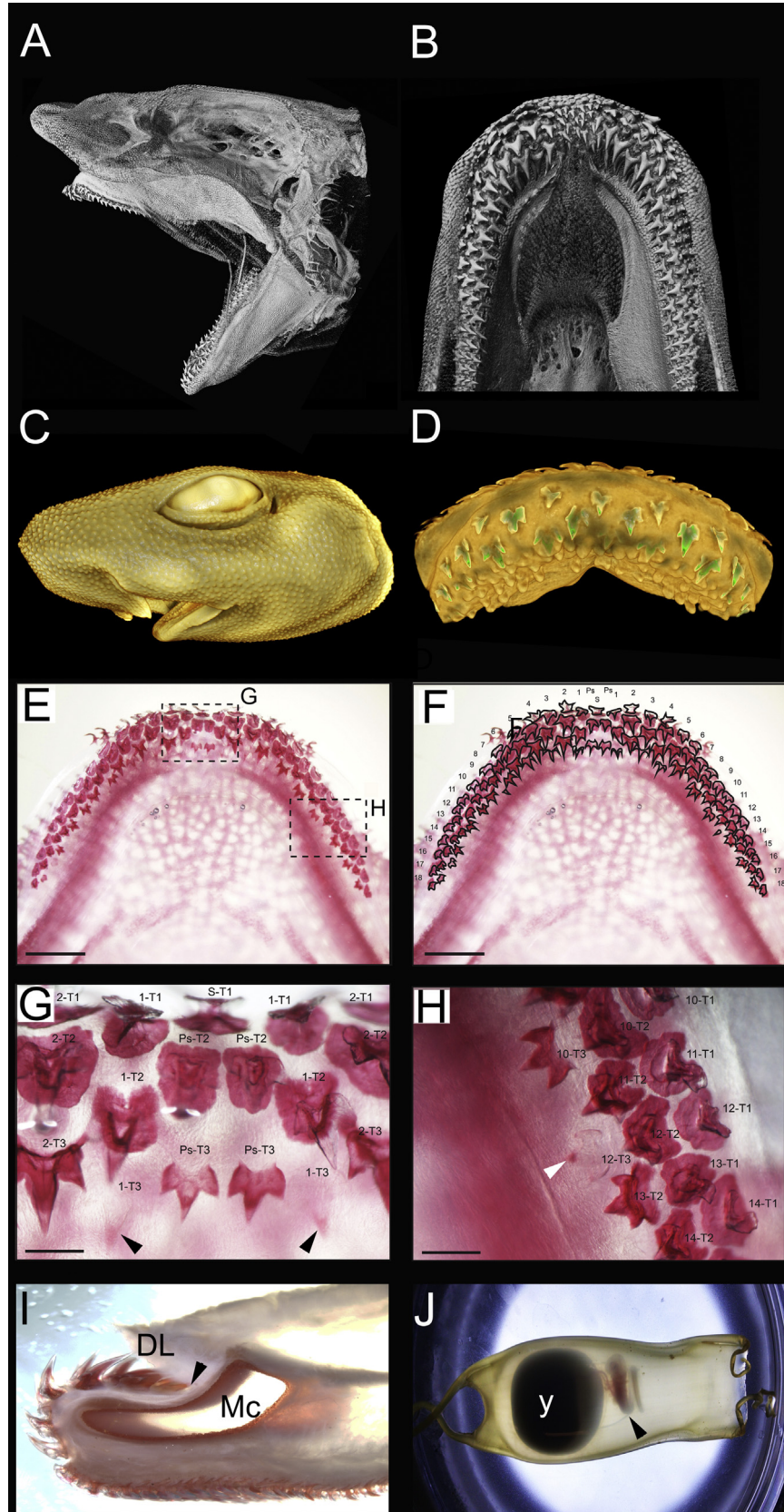
The process of tooth development is highly conserved among bony vertebrates (osteichthyans), from teleost fishes to mammals (Fraser et al., 2009; Smith et al., 2015). Developmental genetic data associated with tooth development and regeneration is growing rapidly in non-mammalian vertebrate taxa. This is particularly apparent in species within the teleost fish lineage e.g. trout (Fraser et al., 2004, 2006b), medaka (Debiais-Thibaud et al., 2007; Gibert

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et al., 2010), cichlids (Fraser et al., 2008, 2013, 2009; Streelman and Albertson, 2006; Streelman et al., 2003), pufferfish (Fraser et al., 2012), and the zebrafish (Jackman et al., 2004; Jackman and Stock,

2006; Stock et al., 2006). These data offer tantalizing insights into the developmental diversity and evolution of osteichthyan dentitions (Smith, 2003; Tucker and Fraser, 2014). In teleost fishes with



multiple generations of teeth (polyphyodonty; e.g. cichlids), the successional regeneration of teeth follows a generational pattern from simple to complex tooth shape (Sire et al., 2002), with adult tooth shapes developing only after several rounds of tooth replacement (Fraser et al., 2013). It has been reported that this shift in dental complexity follows a general trend in tooth morphogenesis evolution (Handrigan and Richman, 2011) and in some species this increased plasticity of tooth phenotype could be governed through the process of regeneration, especially in polyphyodont species (Fraser et al., 2013; Jernvall and Thesleff, 2012).

Despite diversity in form and function, a major finding of this wider research is that the molecular basis of tooth development, including the reciprocal interactions between odontogenic (neural-crest derived) mesenchyme and a competent epithelium, and many of the genes involved in this coordinated cross-talk (Thesleff and Sharpe, 1997; Tucker and Sharpe, 2004; Zhang et al., 2005) is a highly stable process; this conservation has been maintained for approximately 450 million years of vertebrate evolution (Fraser et al., 2004; Hariharan et al., 2015; Smith et al., 2015). This deep developmental conservation of morphogenetic mechanisms and gross evolutionary stability of a dental gene regulatory network suggests that diversity in vertebrate dentitions, whether tooth shape, number, or regenerative potential must be related to repeated regulatory ‘tinkering’ (Jacob, 1977; Lieberman and Hall, 2007) of the same core conserved gene network in different lineages (Bei, 2009; Fraser et al., 2013; Jernvall and Thesleff, 2012).

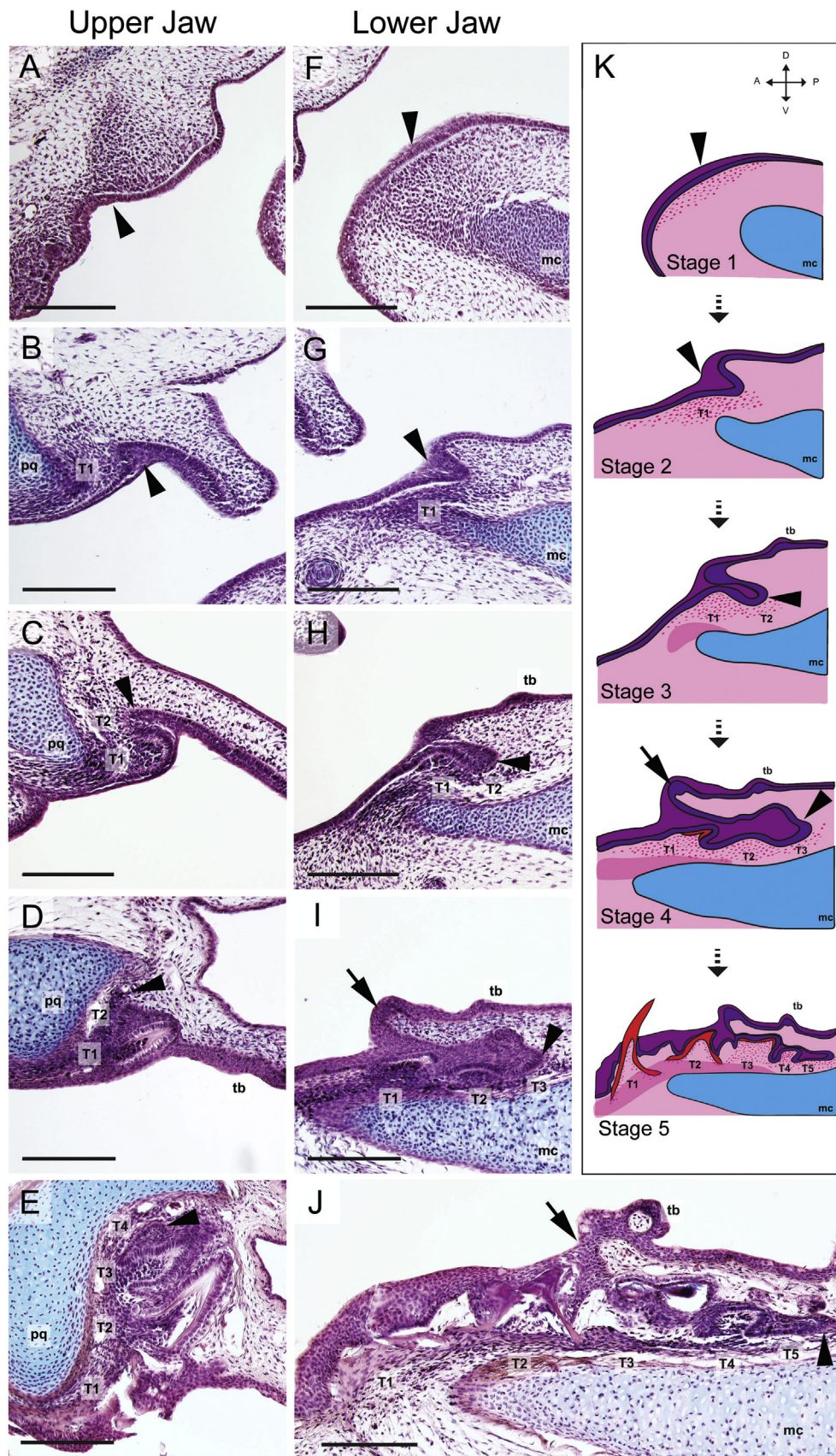
Sharks and their dental characteristics have been discussed and documented for centuries. Owen (Owen, 1840–1845, 1866) was one of the first prominent scientists to appreciate the regenerative capacity of the distinctive predatory shark dentition, describing it as “numerous teeth ever marching slowly forward in rotary progress”. The dentitions of sharks (and rays) are well known for their ability to regenerate in a continuous conveyor-belt manner throughout life, another example of polyphyodontism (Smith, 2003; Smith et al., 2009a; Smith et al., 2009b; Tucker and Fraser, 2014; Underwood et al., 2015). Importantly, evidence from the fossil record suggests that polyphyodonty is not only plesiomorphic within chondrichthyans (Botella, 2006; Botella et al., 2009) but is also the ancestral condition of all vertebrate dentitions (Maisey et al., 2014; Rucklin et al., 2012). Tooth regeneration in chondrichthyans (cartilaginous fishes) can therefore be directly compared with polyphyodont bony vertebrates to determine ancestral versus derived characteristics of the dentition in each lineage and to develop a more complete picture of tooth development and regeneration in the ancestor of all extant vertebrates. Whilst indispensable for ancestral state reconstructions, elasmobranch dentitions also exhibit several derived characteristics (Botella, 2006). Extant holocephalans have dispensed with iterative tooth replacement in favour of the development of more derived, fused tooth plates (Finarelli and Coates, 2012). The sharks and rays (elasmobranchs) however, exhibit a huge diversity with respect to

both craniofacial morphology and the dentition (Cappetta, 2012). Furthermore, although polyphyodonty is the plesiomorphic condition in sharks as in all vertebrates, the dentition of elasmobranchs exhibits regenerative coordination and capacity to a degree unlike any other vertebrate group with the rapid, synchronous production of new teeth from multiple families simultaneously, ahead of functional need (Fig. 1; Luer et al., 1990; Reif et al., 1978). In some species, each round of tooth replacement can be as rapid as 9 (Nurse shark, *Ginglymostoma cirratum*) to 38 days (Leopard shark, *Triakis semifasciata*) (Luer et al., 1990; Reif et al., 1978; Smith et al., 2009a). Therefore, sharks likely possess the most productive and rapid dentition of all vertebrates. Shark teeth are patterned in multiple distinct tooth families (Fig. 1) with numerous teeth formed ahead of function, giving sharks their notorious rows of backward pointing teeth. In sharks, a more rudimentary first generation dentition is thought to form during embryogenesis (Fig. 1) prior to more adult-like tooth morphologies that develop after several rounds of tooth replacement, (Reif et al., 1978; Smith et al., 2009a). This appears to confirm the condition from the majority of current evidence in other vertebrates, which suggests that complex tooth shapes can be formed and functional from first generation teeth in many groups.

Given recent experimental advances in working with chondrichthyans (Coolen et al., 2008; Gillis et al., 2012; O’Shaughnessy et al., 2015), increasing availability of genome-scale data (Mulley et al., 2014; Richards et al., 2013; Takechi et al., 2011; Venkatesh et al., 2014; Wyffels et al., 2014) and the wealth of knowledge on conserved genes that regulate tooth development among vertebrate clades (Fraser et al., 2004; Jernvall and Thesleff, 2012; Smith et al., 2015; Tucker and Fraser, 2014) it is surprising how little is known about tooth development and regeneration in chondrichthyans, which includes sharks, skates and rays (elasmobranchs) as well as chimaerids (holocephalans). Chondrichthyan fishes are the sister group to the bony vertebrates (actinopterygians plus sarcopterygians) and although they have historically been considered primitive compared to the bony vertebrates, this is not a plausible conclusion given recent palaeontological data (Brazeau and Friedman, 2015; Davis et al., 2012; Giles et al., 2015). Recently, the first chondrichthyan genome – that of the elephant shark *Callorhynchus milli* (Holocephali) – was sequenced, revealing extensive conservation of genomic architecture and an extremely slowly evolving proteome compared with bony vertebrates (Venkatesh et al., 2014). Genome sequencing projects of other members of the chondrichthyan clade will be published soon (Coolen et al., 2008; Wyffels et al., 2014) with representative coverage from both the sharks and rays (elasmobranchs). The dissemination of genomic data from these important vertebrates will accelerate their use as models in the study of evolutionary developmental biology.

This study focuses on the catshark (*Scyliorhinus* spp.), an oviparous (Fig. 1) genus that has been widely studied (Coolen et al.,

Fig. 1. The hatchling and adult catshark dentition. A, B. Rendered X-ray computerized tomography (microCT) scans of an adult male *Scyliorhinus canicula*. A. Lateral view of the head; unmineralised tissues are rendered transparent to show jaw cartilages and teeth, with dermal denticles and weakly mineralized chondrocranium also visible. B. Occlusal view of the lower jaw dentition showing multiple rows of teeth and diversity of tooth morphology. C, D. Rendered high-contrast (with Phosphotungstic acid; PTA) X-ray microCT images of the hatchling *S. canicula* head (C), and a section of the lower jaw showing the emerging and functional dentition (D, green), showing multicusp and adult-like tooth shape anteriorly. E–H. Cleared and alizarin red stained hatchling shark (*S. canicula*) lower jaws, showing the maturity and functionality of the earliest shark dentition. E and F, dorsal view of the lower jaw (dentary) showing the numbered tooth positions inclusive of symphyseal tooth positions (F). G, H. Positional shifts in early tooth morphology is noted along the jaw, from more adult-like tooth shape (three defined cusps, with central cusp first to mineralize, arrowheads) in anterior positions, including the medial positions of symphyseal teeth (G) compared to the lack of defined shape in the more distal regions of the jaw (H, although T2, T3 replacements do show the typical three cusps with the central cusp first to form, white arrowhead). This distal most region of the jaw (H) is a dynamic region where serially added new first generation tooth positions are formed in concert with the growth of the jaw. Positions are numbered to reflect positions in the jaw, increasing distally. Tooth generations are numbered in lingual order (T1, T2, etc. S, symphyseal teeth; Ps, parasymphyseal teeth). I. Sagittal thick section (cleared and alizarin red stained) through a juvenile shark (*S. canicula*) lower jaw (Mc, Meckel’s cartilage) showing a mature set of at least 6 teeth of the same family. Replacement teeth are in various stages of development with the youngest replacement teeth in the distal most region (arrowhead) of the dental lamina (DL). Developing teeth are covered by the dental lamina until they erupt at the jaw margin and so contact the region of the external skin with dermal denticles present. J. The developing shark embryo (arrowhead) with associated yolk sac (y) in its clear fluid-filled collagen egg case highlighting the accessibility of this species for developmental biology. Scale Bars in E and F = 1 mm. Scale bars in G and H = 200 μ m.



2008; Debiais-Thibaud et al., 2007, 2011; Freitas and Cohn, 2004; Freitas et al., 2007; Gillis et al., 2012, 2013; O'Neill et al., 2007; Reif, 1980; Smith et al., 2009a) and for which, due to the relative ease of egg-case collection and culture in laboratory aquaria, all embryonic stages of tooth development and regeneration can be manipulated. In this report we outline the emergence, development and primary rounds of regeneration of the dentition in the shark (*Scyliorhinus* spp.) and characterize for the first time the expression patterns of key members of gene regulatory networks and signalling pathways known to be important in the development of osteichthyan dentitions. We ask if tooth development is conserved from a developmental genetic standpoint, both between osteichthyan and chondrichthyan dentitions, and also between subsequent tooth generations of the regenerating conveyor-belt dentition of sharks. We further address the question of whether there is an ontogenetic shift in tooth cusp morphology in sharks that is linked to both the maturation and regenerative potential of the developing dental lamina (Smith et al., 2009b), and whether this process is conserved across the other vertebrate lineages (Tucker and Fraser, 2014). We have therefore examined the morphology of the earliest generations of the catshark dentition, and compared these first tooth generations with the mature dentition to ask if first teeth in sharks are rudimentary and simple in form compared to more complex tooth phenotypes in adult forms. We hypothesize that for a continued process of tooth regeneration, a dental lamina is necessary and associated with the lifelong maintenance of a putative stem cell population. However, the dental lamina is not necessary for teeth, *per se*, as the first generation dentition in several vertebrates, albeit rudimentary and superficial, can form prior to dental lamina formation. We infer that the key character for a highly regenerative dentition, such as that observed in the shark, is a continuous dental lamina, with a non-restricted (not attached to a bony jaw) dentition. Furthermore the rapid regeneration of teeth afforded to the sharks allows more morphogenetic plasticity, formation of diverse tooth phenotypes, and rapid developmental turn-over, which has been essential to the success of this diverse group of cartilaginous fishes.

2. Materials and methods

2.1. Fish husbandry

The species of shark used in this study include the small (lesser) spotted catshark, *Scyliorhinus canicula*, and the large (greater) spotted catshark, *Scyliorhinus stellaris*. The large spotted catshark (*S. stellaris*) was used in this study due to availability of the earliest stages of tooth competence, and to account for the issues in acquiring the correct stages in *S. canicula*. Animals were collected and supplied by the Native Marine Centre, UK (*S. stellaris*) and Station Biologique de Roscoff, France (*S. canicula*). Shark embryos in egg

cases (*Scyliorhinus* spp. is oviparous; Fig. 1) were collected from gravid females and kept and raised to the desired developmental stages (Ballard et al., 1993; Reif, 1980) in a saltwater aquarium at the University of Sheffield, Department of Animal and Plant Sciences at 12 °C. Prior to analysis catshark embryos were anesthetized with MS-222 (tricaine) before fixation in 4% paraformaldehyde (PFA) overnight followed by graded dehydration into MeOH.

2.2. Clearing and staining

Embryos previously fixed in PFA were washed with DEPC-H₂O for thirty minutes. Specimens were then placed into a 1% trypsin solution for one hour. After protein digest, calcified tissues were stained using Alizarin red S solution (1 g/50 mL KOH). Staining averaged 30 min, with larger specimen requiring a longer stain time. Embryos were moved to a 2% KOH solution for a period of 24 h. Cleared and stained fishes were then graded into 100% glycerine, with thymol as a biocide.

2.3. Serial thin sectioning

Embryonic shark heads were fixed in PFA 4% and transferred through a graded series of MeOH (25%, 50%, 75%, 100%) before being transferred to paraffin grades for embedding. Thin serial sections were cut from whole heads at a thickness of 10–14 µm with a Leica Microtome. Thin sections were washed in Histoclear (National Diagnostics) or Xylene before rehydration through graded EthOH washes to PBS (Phosphate Buffered Saline) for immunohistochemical and *in situ* hybridisation analyses.

2.4. Immunohistochemistry

Embryos were fixed (4% PFA/PBS) for 24 h, followed by dehydration through a graded series of ethanol. Specimens were then further processed through a graded series of ethanol, chloroform and hot wax, prior to final embedding in paraffin. Specimens were then sectioned at 14 µm using a microtome (Leica RM2145). Paraffin section immunohistochemistry was carried out using an anti-PCNA antibody (ab29; Abcam) and peroxidase-labelled anti-mouse IgG (DAKO). The colour reaction was carried out using DAB (DAKO) in accordance with the manufacturer's instructions. Tissue sections were counterstained using methyl green or DAPI, and cleared and mounted using DePeX mounting medium (VWR). Sections were imaged using an Olympus BX51 Upright Compound Microscope and Olympus DP71 Universal digital camera attachment.

2.5. *In situ* hybridisation

Digoxigenin-labelled antisense riboprobes were designed using partial skate (*Leucoraja erinacea*) and catshark (sometimes referred to as dogfish; *Scyliorhinus canicula* or *Scyliorhinus torazame*) EST

Fig. 2. The stages of tooth development and replacement in the shark. A–E, Haemotoxinilin and eosin stained thin section paraffin histology (sagittal plane) of upper jaw tooth development and regeneration in the catshark (*Scyliorhinus canicula*). F–J, Haemotoxinilin and eosin stained thin section paraffin histology (sagittal plane) of lower jaw tooth development and regeneration in the catshark (*S. canicula*). K, schematic diagrams of the 4 stages of early tooth development in the shark lower jaw. A, Upper and F, lower jaw histology; Stage 1 indicates the point at which the epithelial odontogenic band thickens (arrowheads) to start the tooth development cascade, with the underlying mesenchyme beginning to condense at tooth competent regions. B and G, the thickened odontogenic band proliferates to become the early dental lamina (arrowheads). The first tooth (T1) starts to form at this stage (Stage 2) with responsive underlying condensing mesenchyme (speckled pink in K) between the dental epithelium and the jaw cartilages (blue). Stage 3 (C and H) is the phase of development where the first tooth in the dentition takes shape, during the differentiation and subsequent morphogenetic period of odontogenesis. While the first tooth starts the process of mineralization, the lingual aspect of the dental lamina (free end, successional lamina) continues to grow and proliferate into the underlying mesenchyme (arrowhead). Stage 4 (G and I) marks the phase of odontogenesis where tooth replacement matures and a number of tooth generations (three generations in the lower jaw, I; T1–3) are formed from the free end (successional lamina) of the permanent dental lamina (arrowheads). There is a pronounced junction within the surface oral epithelium (arrow) that combines cells that will be recruited into the dental lamina with superficial cells of the taste bud territory (tb). Stage 5 marks the time point (hatching stage 34) where teeth of the first generation become functional and erupt through the oral epithelium (T1). At least 5 tooth generations are present at this stage (T1–5) with new teeth initiating from the deep successional lamina (arrow). Teeth are anchored by root-bases to the stratified fibrous cells of the connective tissue envelope that surrounds the jaw cartilages (dark pink in K). Scale bars= 100 µm. mc, Meckel's cartilage; pq, Palatoquadrate. Orientation axes, D, dorsal; V, ventral; A, anterior; P, posterior.

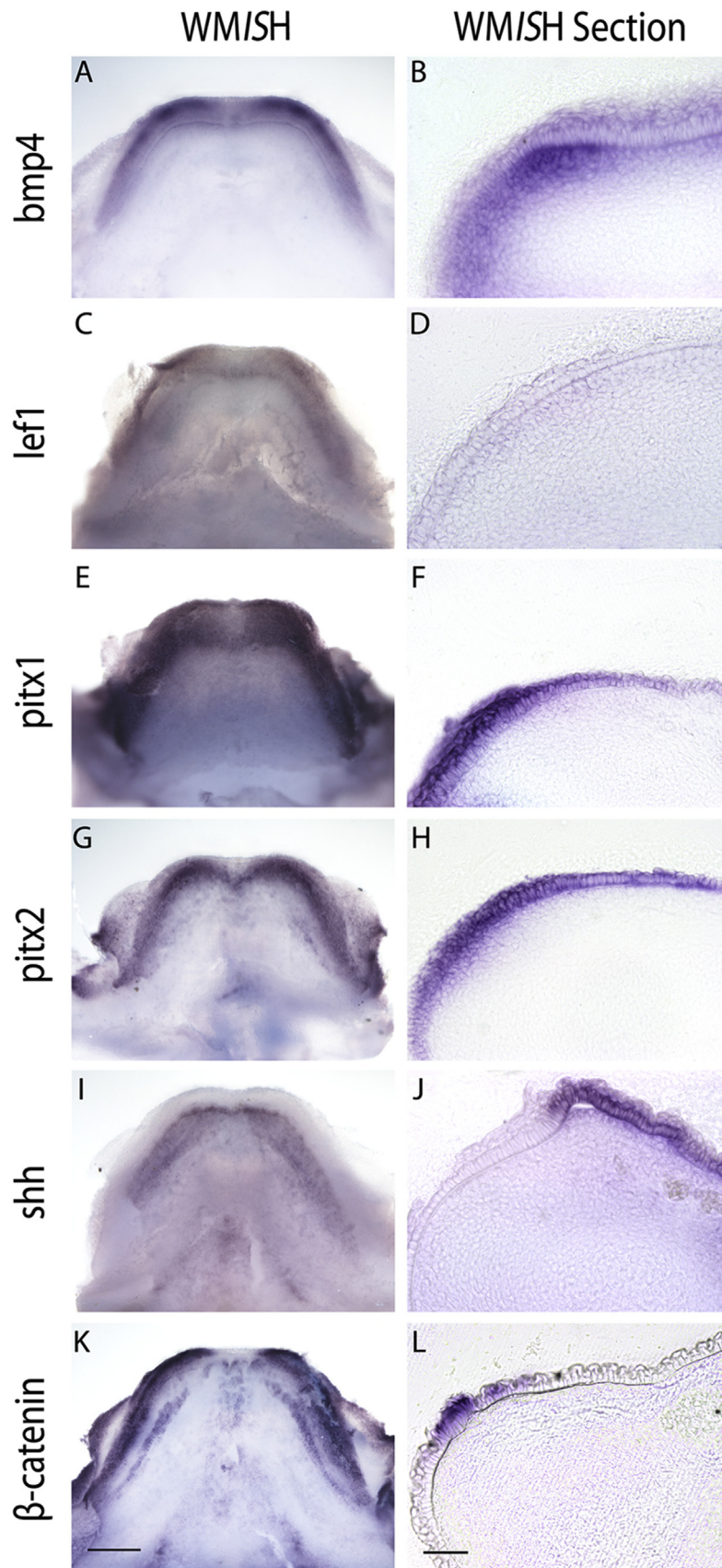


Fig. 3. Formation of the odontogenic band preceding tooth initiation in the lower jaw of the catshark (*S. canicula*). Conserved epithelial and mesenchymal markers of vertebrate tooth development can be observed demarcating the odontogenic band (OB) in the lower jaw of stage 29 catshark embryos (Ballard et al., 1993). Whole mount *in situ* hybridization followed by vibratome sectioning revealed weaker epithelial and stronger mesenchymal expression of *bmp4* (A, B) and weak mesenchymal expression of *lef1* (C, D), underlying the competent OB. Epithelial expression of *pitx1* (E, F), *pitx2* (G, H), *shh* (I, J), and β -catenin (K, L) marks the diffuse competent odontogenic band (OB). Interestingly, *pitx2*, a marker of epithelial dental competence in several vertebrates is expressed here in the shark in both epithelial and mesenchymal domains (H). *shh* and *pitx2* are markers of tooth competence in other vertebrates (Fraser et al., 2008, 2012, 2004; Jackman et al., 2004; Jussila and Thesleff, 2012; Wu et al., 2013), suggesting deep conservation of these core dental markers. Scale bars: (K) 200 μ m, (L) 100 μ m.

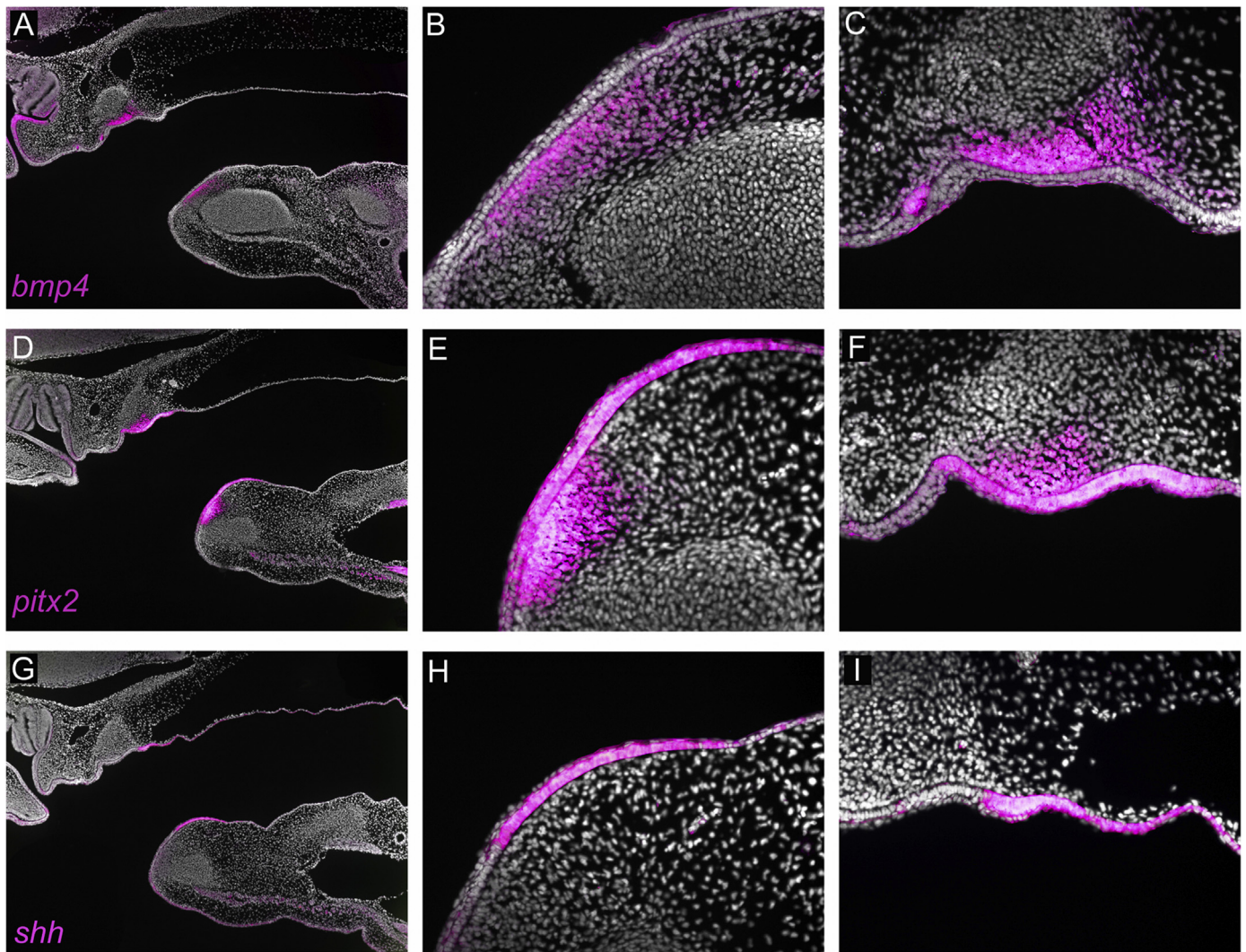
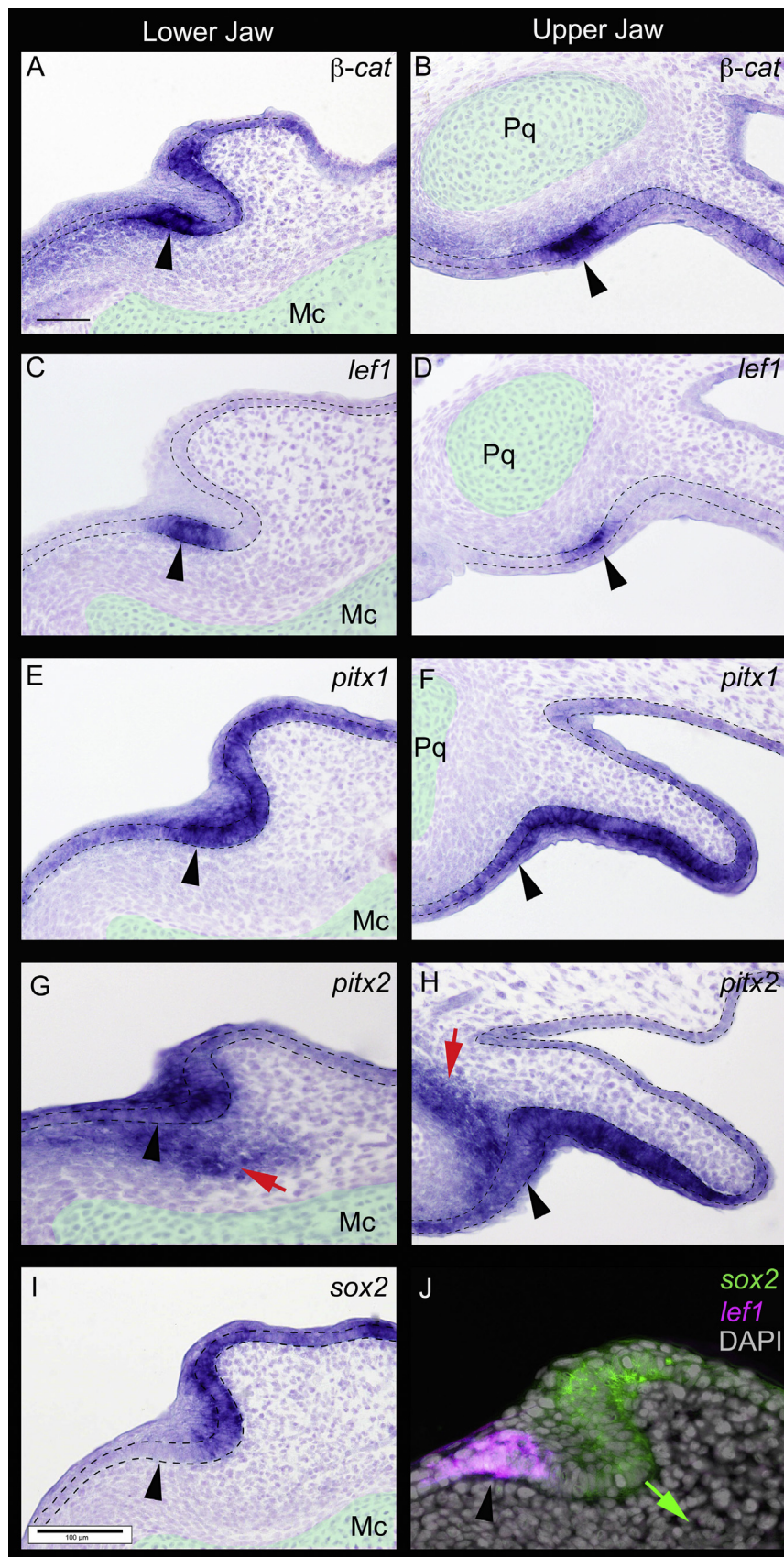


Fig. 4. Shark tooth site pre-patterning with ancient core markers of dental competence. *bmp4*, *pitx2* and *shh* expression patterns (false colour, magenta, *in situ* hybridization) in the shark odontogenic band (*S. stellaris*). A–C, *bmp4* is expressed primarily in the condensing mesenchyme, both in the upper and lower jaws. Localisation of *bmp4* to this mesenchyme, directly underlying the thickened epithelium, in both the upper (C) and lower (B) jaws, implies a role in regulating early odontogenic mesenchymal induction. This is therefore in partial agreement with *bmp4* expression in the mammalian dentition, which at similar tooth stages localises to both the epithelium and mesenchyme (Vainio et al., 1993). D–F, during early priming of odontogenic tissues, *pitx2* expression is localised to the odontogenic epithelium, in addition to underlying expression of the early reciprocal condensing mesenchyme. Expression is comparatively stronger in the thickened epithelium in both the upper (F) and lower (E) jaws, implying both epithelial induction and concomitant transfer of odontogenic potential to the underlying prospective dental mesenchyme, possibly to define the future position of the dental lamina. This is most apparent in the comparatively deepened expression domains of *pitx2* in the lower jaw (E). G–I, *shh* is one of the earliest markers of dental competence in vertebrates and here defines the epithelial odontogenic band, shown by the equivalent expression domains in the presumptive dental epithelium of the upper and lower jaws (H and I). *shh* is expressed in the same restricted region of thickened epithelium in the upper (I) and lower jaws (H), further suggesting a role for Hedgehog signalling in early priming of prospective dental tissues. Nuclear counterstain, white is DAPI.

assemblies (Wyffels et al., 2014) (SkateBase; skatebase.org) and the Vertebrate TimeCapsule (VTCap; transcriptome.cdb.riken.go.jp/vtcap). The riboprobes used in this study were cloned from *S. canicula* cDNA using the following primer sequences, sequence databases and in some cases published genbank accession numbers: β -catenin, (forward GGTGAAATGCTTGGGTCT and reverse GGA-CAAGGGTTCCTAGAAGA; genbank accession number: AF393833.1, (Tanaka et al., 2002), *bmp4* (forward TGTGGAGTTCACCGAATTG and reverse GATTCCTGGTAACCGAATGC; SkateBase), *fgf3* (forward CTTGCTCAACAGCTTAAAGTTATGG and reverse CGGAGGAGGCTC-TACTGTG; SkateBase), *fgf10* (forward TGAAGAGTGCTGAAGGTGGTC and reverse ATTGGACTAATGCTTCAGGTGTG; SkateBase), *lef1* (forward GGGCTTCTGCTGACTGATG and reverse CGTAAGGAGCGG-CAACTTC; VTCap), *midkine* (MK; forward GA-CAGGGTCTCTGAAGCTG and reverse TTAGGGTTCCATTGCGAGTC; VTCap), *pax9*, (forward GCTCCATCAGCAGAATCCTC and reverse TGCCATCACACTTCTTGCTC; genbank accession number:

KC507188.1 (Onimaru et al., 2015), *patched2* (*ptc2*; forward GCGTCGCTGGAGGGTACTT and reverse ATGTCTGTAAAGCA-CAGCCCA; genbank accession number: EU814484.1 (Sakamoto et al., 2009), *pitx1* (forward GGTGGGTGAAAGCAGAG and reverse GATGTTGCTGAGGCTGGAG; SkateBase), *pitx2* (forward GACG-GAAGCTGGAACAGTC and reverse TTTGCAAAGTGGGTGTCAAG; genbank accession number AB625610.1 (Takechi et al., 2011), *sonic hedgehog* (*shh*; forward AGTGGCAGATACGAAGGGAAG and reverse AGGTGCCGGAGTACCAG; genbank accession number: HM991336.1 (Gillis et al., 2011), and *sox2* (forward GGAGTTGT-CAGCCTCTGCTC and reverse TGTGCTTTGCTGCGTGTAG; Skate-Base). Whole mount *in situ* hybridisation (WISH; modified for thin section SISH) was performed according to previously published protocols (Fraser et al., 2008; 2013). Digoxigenin-labelled anti-sense riboprobes were generated using the Riboprobe System Sp6/T7 kit (Promega). APconjugated anti-dig antibodies were visualized at the end of colour reaction (BM purple; Sigma) using light/



confocal microscopy.

2.6. Whole mount *in situ* hybridisation (WISH)

Whole Mount *In Situ* Hybridisation (WISH) was carried out in accordance with Fraser et al. (2013). Samples were rehydrated through a graded series of methanol and PBS, and treated with Proteinase K (1 µl/mg ProK for 60 min), to facilitate probe penetration. Next, samples were re-fixed in 4% PFA in PBS and incubated in prehybridization buffer for 1 h at 61 °C. For the hybridization stage, samples were placed in a shaker incubator overnight at 61 °C in 2 ml tubes (Eppendorf) containing 1 ml aliquots of hybridization buffer and DIG-labelled antisense RNA probe. Samples were then washed in Saline Sodium Citrate with 0.1% Tween-20 (SSCT), before incubation in Blocking Reagent (Roche). Antibody labelling occurred overnight at 4 °C in Maleic Acid Buffer with Tween-20 (MABT), using anti-DIG-ALP (0.2 µl/ml) (Roche). This was followed by a series of washes and 48 h incubation in MABT at 4 °C. For the colour reaction, BM Purple (Roche) was applied at room temperature, until the staining was sufficiently strong to accurately represent gene expression. Samples were stored and imaged in 10% EtOH in PBS. After whole mount *in situ* hybridisation and imaging, embryos were embedded in chick albumin with gelatin cross-fixed with 2.5% glutaraldehyde and post-fixed with 4% PFA. A Leica Microsystems VT1000 vibratome was used to cut sections at 15–25 µm. Vibratome sections were then mounted with glycerol and imaged at 10–63× using a Nikon SMZ1500 Stereomicroscope.

2.7. Section *in situ* hybridisation (SISH)

Specimens were first processed to paraffin and sectioned as previously described. Thin serial paraffin sections were re-hydrated from MeOH and ISH was carried out on paraffin sections with solutions described in WISH. Tissue sections were treated with pre-hybridisation buffer, followed by overnight incubation with probe. Sections were then subject to post-hybridisation washes and incubated with an AP-conjugated anti-dig antibody overnight. Sections were then subject to post-antibody washes and the colour reaction carried out using BM Purple (Roche). Sections were then counterstained using haematoxylin (or DAPI) and images taken as previously described.

2.8. X-ray microtomography

A fixed and air dried head of an adult male *Scyliorhinus canicula* (Fig. 1A and B) was scanned using the Metris X-Tek HMX ST 225 CT scanner at the Imaging and Analysis Centre, Natural History Museum, London, and rendered using VG studio max 2.0. High-resolution X-ray microCT images of hatchling *S. canicula* specimens were made at the University of Vienna, Department of Theoretical Biology imaging lab using the soft-tissue contrast methods of Metscher (Metscher, 2009a, 2009b). *S. canicula* specimens were

fixed in 4% paraformaldehyde, then prepared as follows: Specimen in Fig. 1C (108 mM TL) was dehydrated to ethanol and stained with Phosphotungstic acid (1% w/v in abs. ethanol) for 3 days. specimen in Fig. 1D (105 mM TL) was washed in water and stained several days in IKI (2% potassium iodide+1% I₂ in aqueous solution). specimen in Fig. 8A and B (55 mM TL) was dehydrated to ethanol and stained with I2E (1% I₂ in 96–10% ethanol). low-resolution overview scans were made with a SkyScan 1174 system (bruker-microCT.com), and high-resolution images of the lower jaws were made with the Xradia MicroXCT (www.xradia.com). Tomographic images were reconstructed using the SkyScan NRecon program or the Xradia XMReconstructor software and exported as TIFF or PNG image stacks. 3D volume renderings were made using the free software Drishti (github.com/AjayLimaye/drishti). Reconstructed isotropic voxel sizes are 32.2 µm (Fig. 1C), 8.0 µm (Fig. 1D), and 4.8 µm (Fig. 8A and B).

3. Results

3.1. Establishing the dentition in sharks

The shark (catshark; *Scyliorhinus*) dentition initiates at approximately Stage 28–29, depending on temperature of development (Ballard et al., 1993; Debais-Thibaud et al., 2011; Smith et al., 2009a) from a layer of superficial epithelial cells that make up the odontogenic band (OB; Figs. 2A, F, and 3). The OB is a field of epithelial cells that marks a region from which the teeth, coincident dental lamina, and other supporting structures form in sharks, including the closely associated rows of taste buds (Figs. 1C, D, and 2). The wider field of epithelial cells associated with the OB, which encompasses adjacent cells that contribute to developing taste buds (Figs. 2H, I and 3) could be considered an 'odonto-gustatory band' due to their closely linked development. The underlying mesenchymal cells pool and condense beneath the dental competent epithelium to facilitate the proliferation and development of the tooth buds (Figs. 3B, H and 4A–F). These morphogenetic movements, which accompany tooth site initiation from within the OB, are highly conserved among vertebrates (Smith et al., 2009b), and in sharks the dentition is similarly initiated. In the early dentition of the catshark we have examined the expression of a number of genes known to be important to odontogenic band formation and tooth site specification in bony vertebrates. We have found that a number of conserved markers of OB formation are expressed in both the epithelium and underlying mesenchyme in the catshark in a similar manner to other vertebrates (Figs. 3 and 4). We cloned partial catshark mRNA transcripts of six odontogenic epithelial markers and examined their expression in developing catshark jaws *in situ*. We found that *bmp4*, *lef1*, *pitx1*, *pitx2*, *shh*, and β -catenin are expressed within the epithelium of the OB preceding tooth initiation (Figs. 3 and 4). Both *shh* and *pitx2* are among the earliest epithelial markers of tooth competence known in bony vertebrates (Fraser et al., 2008, 2012, 2004;

Fig. 5. Tooth initiation and dental lamina emergence in the early shark dentition. Gene expression patterns during shark (*S. canicula*) tooth initiation. A, C, E, G, I, J, lower jaw; B, D, F, H, upper jaw. During development of the dental lamina from a thickened epithelium (lower jaw, IJ), β -catenin (β -cat) is strongly expressed in the epithelium of first tooth placode (A, arrow) and a cluster of epithelial cells at the DL-oral surface interface via an extension of the ODE (A, arrow). In the upper jaw (B), β -catenin is also strongly expressed in the thickened odontogenic band epithelium (B, arrow) and weakly in the neighbouring oral epithelium (B, dotted line). A, In the lower jaw, *lef1* is expressed primarily in the epithelium of the first tooth placode (arrow), coincident with β -cat expression (A), and in the upper jaw, the early first dental thickening (D, arrow). *pitx1* is expressed throughout the DL, inclusive of the site of the first tooth placode (E, arrow), extending into the lingual DL and adjoining dental epithelium (E, dotted line). Expression is similar in the upper jaw, localised to the early dental epithelial thickening/DL (F, arrow) and extending to within the medial valve (finger-like projection, in F and H) via the epithelium (F, dotted line). *pitx2* is expressed in a similar pattern in the dental epithelium (G, black arrowheads), however; in addition, expression extends to the underlying dental mesenchyme in both the upper and lower jaw (G–H, red arrows). I and J, *sox2* gene expression in the shark lower jaw is limited to the non-tooth site epithelial dental lamina at the first tooth emergence. *lef1* (in C and J) as a marker of tooth placode initiation is expressed adjacent to *sox2* in the emerging dental lamina. J, the expression of *lef1* and *sox2* shows the exclusive sites of each marker, *lef1* (false colour, magenta) in the dental epithelium (first tooth initiation site) and *sox2* in neighbouring non-tooth specific and proliferative dental lamina (false colour green, and predictive growth of the DL shown as green arrow). White nuclear stain is DAPI. Mc, Meckel's cartilage. Pq, palatoquadrate cartilage. Scale bar in A, 50 µm and I, 100 µm.

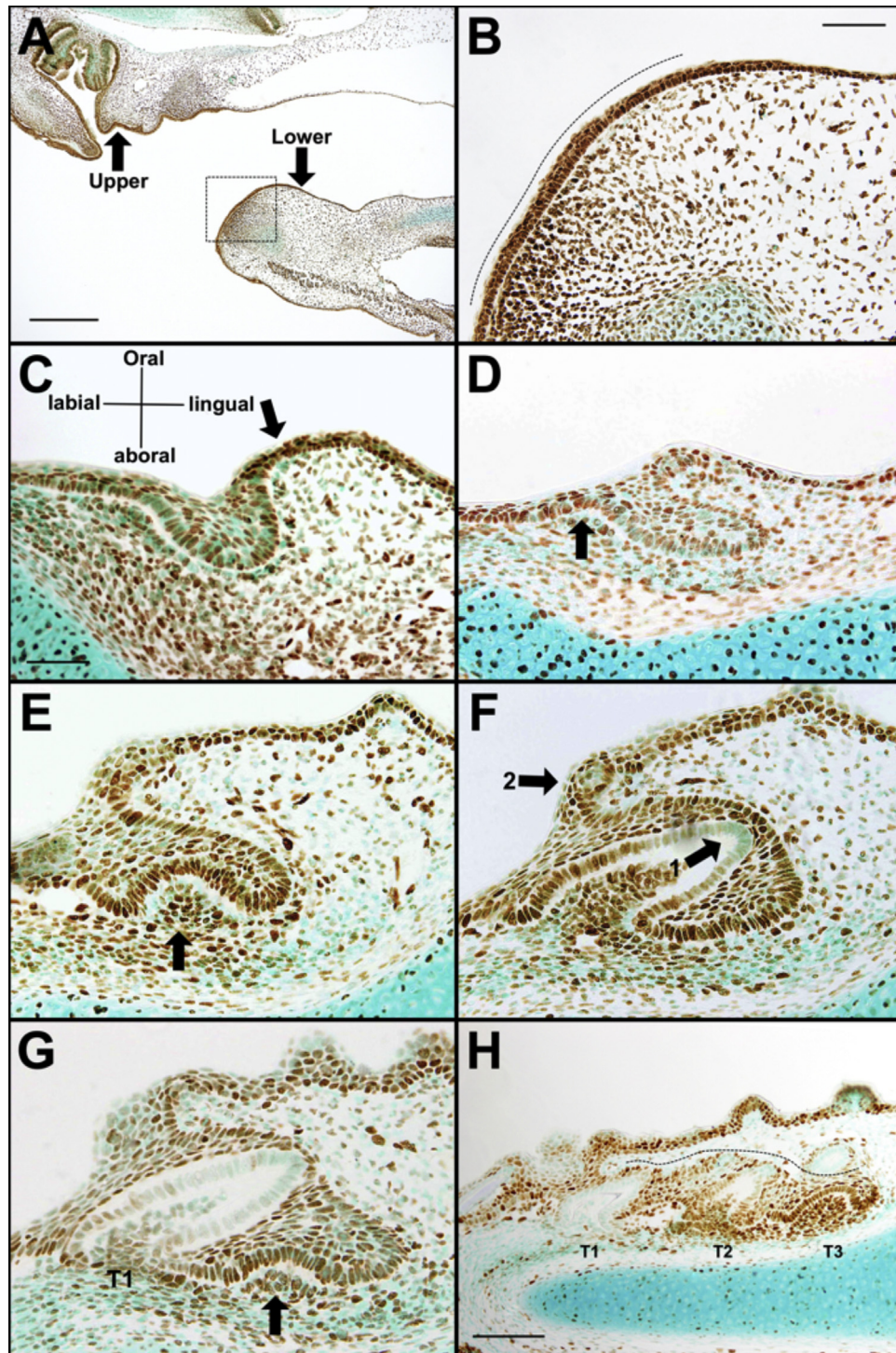


Fig. 6. Proliferating Cell Nuclear Antigen (PCNA) Immunohistochemistry of lower jaw tooth development in the shark. A-B, *S. stellaris*; C-H, *S. canicula*. In the lower jaw oral margin (A, boxed area), PCNA immunoreactivity marks initial epithelial thickening and associated mesenchymal condensation to define early dental competence (odontogenic band) (B, dotted line). Development of the primary dental lamina, PCNA continues to mark epithelial in-folding into the condensing dental mesenchyme, biased to the lingual aspect (C, arrow). D, development of first generation teeth, PCNA marks the first tooth placode, accompanied by further extension of the dental lamina (arrow). E, the first tooth germ forms from epithelial cells of the dental lamina and the in-pushing of the condensing dental mesenchyme (arrow). During morphogenesis, epithelial-mesenchymal cells differentiate to ameloblasts and odontoblasts, marked by reduced PCNA immunoreactivity in the apical tooth (F, arrow 1). The first replacement tooth placodes (G, arrow) develop in the continuously proliferating successional lamina, lingual to advancing first generation teeth (T1). Emergence of tooth replacement, PCNA immunoreactivity in the successional lamina marks third generation teeth (T3, early morphogenesis), progressively reducing in preceding generations undergoing advanced morphogenesis (T2-1). Throughout tooth development and replacement, a continuous connection is maintained between the dental lamina/successional lamina and the surface oral epithelium via the dental epithelium, terminating in a distinct spherical cluster of non-proliferative cells at the junction of the dental lamina and the oral taste territories (F, arrow 2) and (H, dotted line). Scale bars: (A) 1 mm, (B) 100 μ m (C-G) 100 μ m, (H) 200 μ m.

Jackman et al., 2004; Jussila and Thesleff, 2012; Wu et al., 2013), and in the catshark lower jaw, these genes are expressed in an equivalent pattern (Figs. 3 and 4). However, there is a significant

difference in the expression of *pitx2* in the catshark compared to osteichthyans during odontogenesis, with expression observed in a mesenchymal domain directly underlying the sites of epithelial

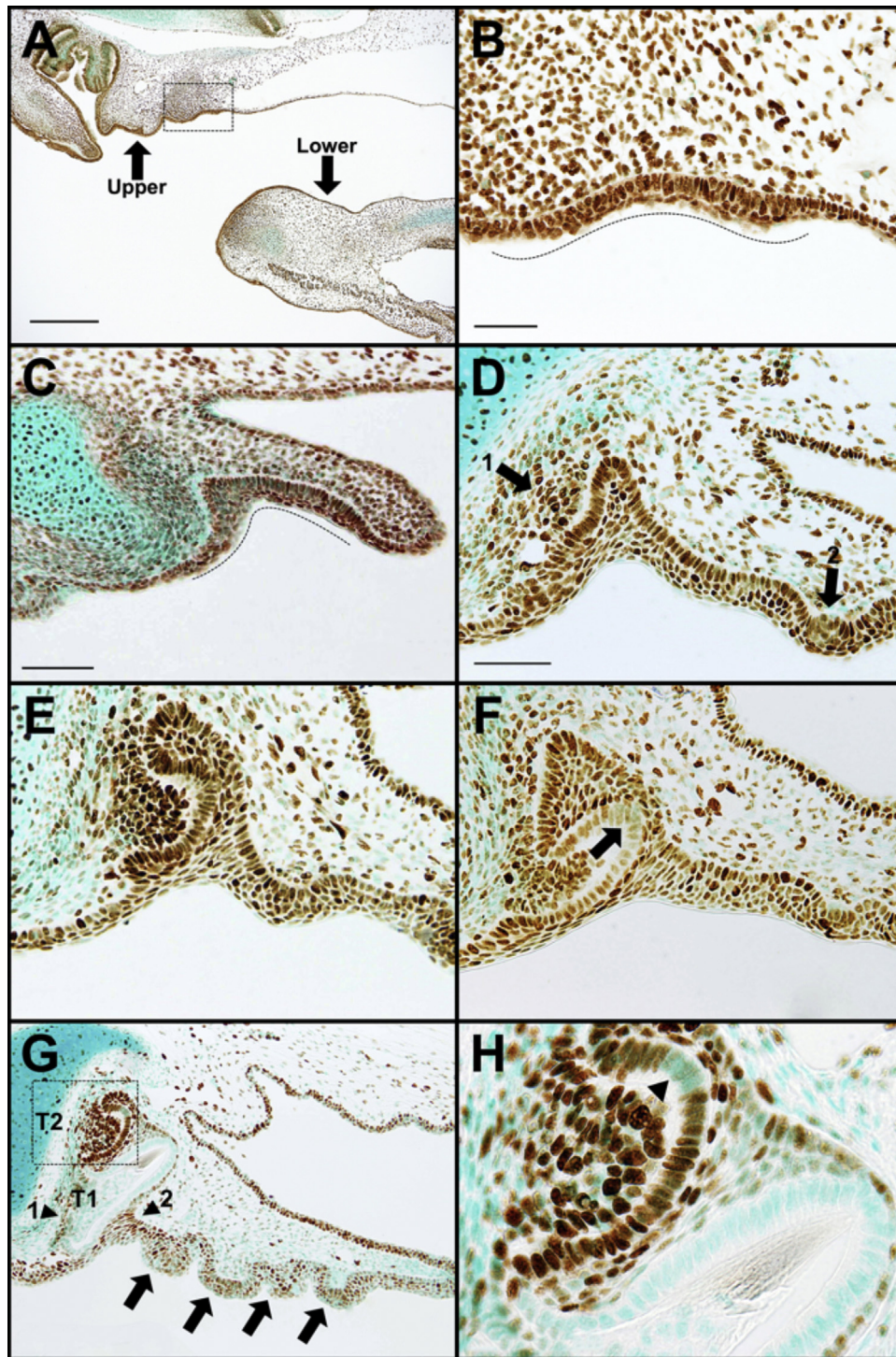


Fig. 7. Proliferating Cell Nuclear Antigen (PCNA) Immunohistochemistry of upper jaw tooth development in the shark. A-B, *S. stellaris*; C-H, *S. canicula*. In the upper jaw, PCNA immuno-reactivity further defines localised thickening of the oral epithelium and condensing mesenchyme to mark onset of dental competence (A, boxed area). A restricted band of thickened columnar epithelial cells mark the odontogenic band, and future position of the prospective dental lamina (B, dotted line). Sustained PCNA immuno-reactivity marks formation of the early dental lamina as this epithelium, continuous with the medial valve, in-folds into the underlying dental mesenchyme (C, dotted line). Development of the first tooth bud is defined by outgrowth of columnar ODE cells, associated with underlying mesenchymal condensates (D, arrow 1). The dental epithelium is also continuous with the basal epithelium of the medial valve, defined by early taste bud papillae (D, arrow 2). During first tooth morphogenesis, PCNA immuno-reactivity continues to show sustained condensation of the underlying dental mesenchyme and early epithelial growth polarity (E). During advanced morphogenesis, reduced PCNA immuno-reactivity in the apical inner dental epithelium and dental mesenchyme of the tooth further implies cell differentiation in advance of matrix deposition (F, arrow). PCNA continues to label the outer dental epithelium and dental lamina during replacement tooth morphogenesis (G, boxed area T2; arrows highlight taste buds on the medial valve), with a corresponding reduction in immuno-reactivity during advanced morphogenesis (T1), accompanied by associated deposition of mineralised tissue. In the basal dental mesenchyme of the papilla, sustained PCNA immuno-reactivity marks continued cell proliferation concomitant with basal plate formation (G, arrow 1), while continuing to define a connection between the dental and oral epithelium (G, arrow 2). During replacement tooth morphogenesis, a nested cluster of cells in the apical epithelial tip (H, arrow) lacks PCNA immuno-reactivity, compared with the surrounding inner dental epithelium and dental mesenchyme. Their implied withdrawal from the cell cycle suggests a functional requirement distinct from surrounding epithelial cells. Similar developmental patterns in the mammalian, osteichthyan and reptilian dentitions imply a deeply conserved functional role in the shark dentition (Fraser et al., 2013; Smith et al., 2009b; Juuri et al., 2013; Gaete and Tucker, 2013). This is particularly apparent in the non-proliferative epithelial tip, which in the mammalian dentition lacks similar cell proliferation due to activation of the enamel knot, a conserved signalling centre regulating tooth cusp morphogenesis (Jernvall et al., 1994; Vahtokari et al., 1996). Scale bars: (A) 1 mm, (B, D, E, F) 100 μ m (C, G) 200 μ m.

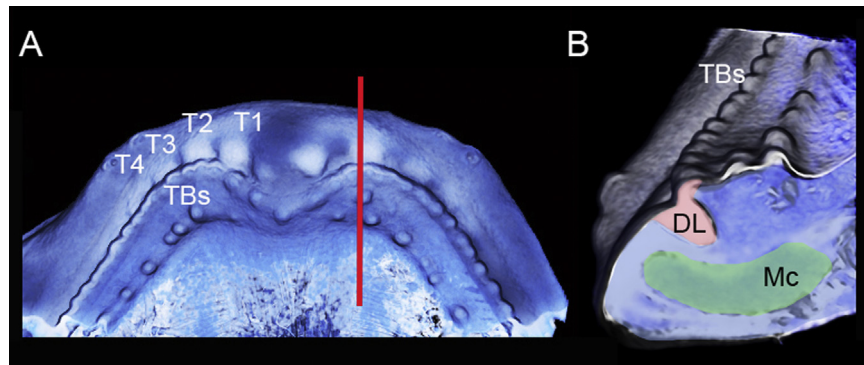


Fig. 8. Soft tissue contrast rendered microCT scan of the pre-hatchling shark lower jaw. Prehatching stage 32 (Ballard et al., 1993); 55 mm TL, *Scyliorhinus canicula* lower jaw, dorsal view, showing the emerging tooth positions of the first generation (T1–4) adjacent to more lingual tooth bud territories (TBs) closely linked to the epithelial cells of the dental lamina that link surface epithelia with deep lying cells of the lamina (B). B, a virtual (microCT) cross section (sagittal plane, red line) of the lower jaw developing dentition with false coloured enhanced soft tissue. Tooth germs are superficial to the epithelium of the dental lamina (pink) that grows toward the underlying jaw cartilage (green, Mc, Meckel's cartilage).

tooth competence (Figs. 3G, H and 4D–F). This expression overlaps with that of mesenchymal *bmp4* (Fig. 4A–C). These data suggest that core components of the gene regulatory network required for the initial establishment of the OB have been conserved in vertebrates for approximately 450 million years. Therefore, we suggest that vertebrates have utilized this dental pre-pattern since the appearance of the first dentition. In concert with the expression of these initiatory factors in the OB, a number of mesenchymal markers, i.e. *bmp4*, *pitx2* and *lef1*, concurrently label the condensing mesenchyme that will delineate the 'mesenchymal dental field' (Figs. 3 and 4) and the future mesenchymal papillae of the developing teeth.

3.2. Emergence of the shark dental lamina

The dental lamina (DL) is required for the development of regenerative multi-generational dentitions in a range of polyphyodont vertebrates, including reptiles, which retain a permanent, thin successional lamina deeply embedded in the jaw (Handrigan et al., 2010; Juuri et al., 2013). Even diphyodont mammals such as ferret (Juuri et al., 2013), and human (Olley et al., 2014) retain an invaginated DL before it eventually degrades after production of the second tooth generation (Buchtová et al., 2012). In contrast, in some polyphyodont fishes, for example the rainbow trout (*Oncorhynchus mykiss*), a deep DL has been shown not to be required for the production of multiple generations of teeth, and replacement teeth can develop from a set of dental cells linked to the outer dental epithelium (ODE) (Fraser et al., 2006a), in association with differentiation of cells of the predecessor teeth. These ODE cells could be interpreted as a basic 'lamina', lying deep to the predecessor for continued tooth production in a 'one-for-one' manner, similar to the replacement system observed in cichlids (Fraser et al., 2013). It is currently uncertain whether oral teeth in the vertebrate ancestor were initiated from a permanent deep DL as observed in polyphyodont amniotes, or from a transitory epithelial thickening, as observed in some ray-finned fishes. In the catshark, a deep permanent DL is formed within which teeth of the first, and all subsequent replacement generations develop (Fig. 2B–E and G–K). The catshark DL first emerges as a highly dynamic epithelial structure that forms after the proliferation of superficial epithelial cells of the OB. The DL is comprised of a monolayer of distinctly columnar basal epithelial cells (future inner dental epithelium), and an overlying (5–6 cell thick) layer of squamous/cuboidal epithelial cells (future ODE) (Fig. 2).

β -catenin, *sox2* and *pitx1*-expressing cells label the early emerging DL, lingual to the cells that contribute to the superficial first tooth (Fig. 5A, E, I and J). The highly proliferative nature of the

emerging dentition and the development of the DL is highlighted by PCNA (Proliferating Cell Nuclear Antigen) immunohistochemistry (Figs. 6 and 7). PCNA marks several phases of the cell division sequence from late G1 through to mitosis (Kurki et al., 1986). The primary stages of first generation tooth initiation and the expansion of the early DL are marked by high rates of proliferation (PCNA; Figs. 6 and 7). Prior to DL invagination, strong PCNA immunoreactivity uniformly marks all cells of the thickening oral epithelium comprising the OB including basal columnar and superficial squamous cells of the presumptive/emerging DL (Figs. 6B and 7B). This apparent proliferative population of epithelial cells could potentially feed the DL as it invaginates and grows into the underlying jaw mesenchyme (Figs. 6C–E and 7C–F), toward the underlying cartilages of both the upper and lower jaw. The underlying jaw mesenchyme is also highly proliferative at this stage; however, prior to DL invagination (Fig. 8) there is a notable asymmetry in cell proliferation, with presumptive dental mesenchyme on the labial side of the DL exhibiting a greater density of cells that show proportionally greater PCNA immunoreactivity (Figs. 6C–E, 7D–E). This asymmetry persists into the DL invagination stages (Figs. 6C and 7C). The mesenchymal expression of *pax9*, *pitx2*, and *bmp4* is specific to a deep lying and condensing cluster of mesenchymal cells (Fig. 9), and demarcates the labio-lingual border of the mesenchymal 'path' into which the subsequent dental lamina will grow as the epithelium proliferates and invaginates toward the jaw cartilages (lower, Meckel's and upper, palatoquadrate; Figs. 5–7). This deep mesenchymal cell condensation extends along with the continuous dental lamina around the arc of each jaw. The initial dental lamina is a jaw wide thickening of epithelium that aggregates from the OB and continues to grow as a dynamic sheet of proliferative epithelia from which new tooth generations form at the distal free-end or successional lamina (Figs. 2, 5, and 8).

In the catshark, we observe that the first tooth buds develop superficially prior to the invagination of the deep DL, which only begins to invaginate into underlying mesenchyme after the initiation of the first tooth (Figs. 5, 6D and 7D–F). The site of first generation tooth initiation coincident with the DL is specifically identified by the epithelial expression of β -catenin, and *lef1*, overlapping in the epithelial portions of the broader expression domains of both *pitx1* and *pitx2*, which encompass the whole DL (Fig. 5). PCNA immunohistochemistry reveals a high level of cell proliferation maintained at this stage in both the epithelium and mesenchyme of the nascent tooth bud, and also throughout the whole dynamic region of the developing jaw-length DL (Figs. 6D and 7D). Subsequently, high proliferative activity continues in both the successional lamina and the surrounding, condensing cells of

the underlying odontogenic mesenchyme during lamina extension and replacement tooth development stages. Meanwhile proliferation tapers off in the surrounding non-odontogenic mesenchyme and terminally differentiated ameloblasts of morphogenetic-stage teeth (Figs. 6F, G and 7F–H). Within the condensing mesenchyme, underlying the extending DL, we observed the weak expression of *β-catenin* and *lef1*, in addition to stronger expression of *bmp4*, *pitx2* and *pax9* (Figs. 5 and 9). These expression patterns are mostly consistent with the variety of vertebrates studied to date, with a similar expression of mesenchymal markers, regardless of replacement potential. Interestingly, although *sox2* is expressed in the epithelial OB (data not shown), it is also expressed in the dorso-lingual and non-tooth region during the initial thickening/growth phase of DL development (Fig. 5I and J). This suggests that *sox2* might be associated with patterning tooth competent epithelium (OB and DL) and expansion of the DL, as reported in other vertebrates (Juuri et al., 2013) rather than initiation and development of the tooth itself. The expression of *lef1* at the same stage of odontogenesis (Fig. 5J) within the tooth-specific epithelial cells of the thickened DL shows the contrast of tooth-site expression with *sox2*-positive adjacent lamina cells. The establishment of this embryonic shark dentition not only sets up the functional dentition that the hatchling shark (Stage 32–34; Fig. 1; Ballard et al., 1993) will rely on immediately after emerging from the egg case (Fig. 1D and E), but will also set the programme of tooth replacement that continues throughout adult life. We predict that this continuously active DL will continue to retain proliferative activity at varying levels throughout the lifetime of the shark to regenerate an unlimited supply of precisely arranged tooth families along the jaws.

3.3. The formation of the first teeth in the catshark

The first generation of teeth in the catshark dentition functions

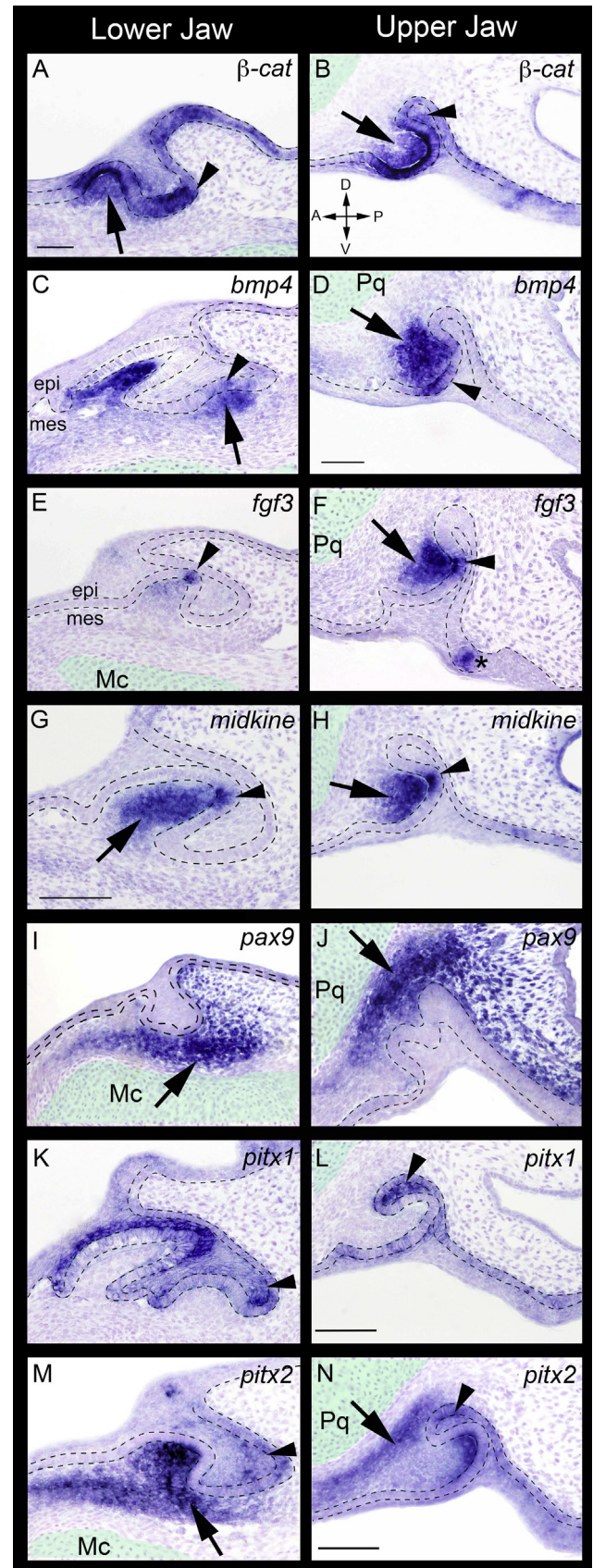


Fig. 9. Development of the first tooth and progression of the dental lamina in the shark (*S. canicula*). Lower jaw (left) and upper jaw (right) sagittal thin sections (14 μ m paraffin sections); dotted lines demarcate the boundary between the basal epithelium and the underlying mesenchymal cells. A and B, *β-catenin* is expressed in both the inner dental epithelium of the developing first tooth and outer dental epithelium in the region of the successional lamina where new tooth replacements will form (arrowhead). *β-catenin* is also expressed in the dental papillary mesenchyme of the developing first tooth (arrow). C and D, *bmp4* has a restricted expression pattern in apical cells of the inner dental epithelium of cap-shaped developing teeth in all generations (arrowheads). *bmp4* is also expressed in condensing mesenchyme associated with new tooth germs of the first and subsequent generations (arrows) and in the maturing dental papilla. E and F, *fgf3* expression marks the enamel knot-like (EK) cells (arrowhead) associated with tooth morphogenesis and shape in sharks and other vertebrates. In addition, *fgf3* expression labels dental papillary mesenchyme directly underlying the EK and the inner dental epithelium. The permanent dental lamina is connected to the surface epithelium at a junction that expressed a small set of *fgf3* expressing cells (asterisk in F). G and H, the heparin-binding growth factor *midkine* (MK) is co-localised with *fgf3* expression, limited to the enamel knot-like cell cluster (arrowhead) of the inner dental epithelium of the first generation teeth, in addition to the underlying mesenchymal papilla (arrow). I and J, *pax9* is an early marker of the dental mesenchyme, and is expressed in the condensing mesenchyme that surrounds the proliferating dental lamina in both the emerging upper and lower dentition; as tooth germs develop *pax9* expression is present in the papillary dental mesenchyme. *pax9* expression demarcates the mesenchymal cells between the developing teeth and the underlying cartilages (arrows; Mc, lower and Pq, upper). *pitx1* and *pitx2* have overlapping gene expression patterns in the dental and successional lamina (K–N; arrowheads). Weak expression of *pitx1* in the majority of the dental lamina is contrasted with the strong expression in the first generation teeth during morphogenesis (K) and in intermediate epithelial cells of the successional lamina related to new subsequent tooth formation, co-expressed with *pitx2* (arrowheads). *pitx2* in the shark has an alternative expression pattern to other vertebrates studied with expression in cells of the dental mesenchyme co-localised with the expression of *pax9* (I and J), with expression in the dental papilla and basal mesenchyme adjacent to the underlying developing jaw cartilages (arrows) and close to the successional lamina. Mc, Meckel's cartilage; Pq, palatoquadrate; epi, epithelium; mes, mesenchyme. Scale bars in A and D = 100 μ m (same magnification for A–F, H, K). Scale bars in G, L, and N = 100 μ m.

as place-markers for the emergence of subsequent generations within each tooth family. These families will eventually form the fully functional dentition comprised of multiple rows of erupted teeth once the permanent regenerative lamina is established. In the catshark (*Scyliorhinus*), we observe medio-lateral initiation of teeth (Tooth position 2; Figs. 1 and 8; e.g. Smith et al., 2009a), with the first teeth forming on either side of the symphysis with one central cusp (first to form; Fig. 1D and G) plus additional cusps that show morphogenesis toward becoming tricuspid. More lateral teeth along the first generation row show more irregular shape: the central cusp is sometimes pointed, and the accessory cusps are generally not pointed, if present at all (Fig. 1E–H). The extreme examples of this are observed in tooth positions closest to the upper/lower jaw joint showing basic triangular cusps or amorphous teeth overlying the dentine root-base (Fig. 1H), contrasting with the obviously multicuspate teeth seen in this position in adults (Fig. 11). Furthermore, up to the hatching period, even subsequent generations (G2, G3 at least) produce exactly 3 definitive cusps, whereas in a juvenile/adult most teeth are pentacuspate (5 definitive cusps; Fig. 11). However, the exceptions are the minuscule symphyseal teeth and the very newest tooth families that are added to the distal margins of the jaw as the shark grows. These teeth are each tricuspid, as in the embryo/hatchling (Fig. 1G). These data show that the teeth of the first generation in *Scyliorhinus* are rudimentary and irregularly shaped, with mostly incomplete morphogenesis compared to subsequent generations of teeth. Even subsequent generations (G2, G3) form fewer cusps (tricuspid) than a juvenile/adult (pentacuspate) (Figs. 1 and 11). Regeneration is therefore necessary in the catshark not only later in life for functional tooth replacement, but also in early development to progressively establish the adult-type functional tooth morphology.

At hatching, the full regenerative dentition of *S. canicula* is comprised of up to 5 generations of teeth in the medio-lateral lower jaw tooth region. Up to three of these generations are under-

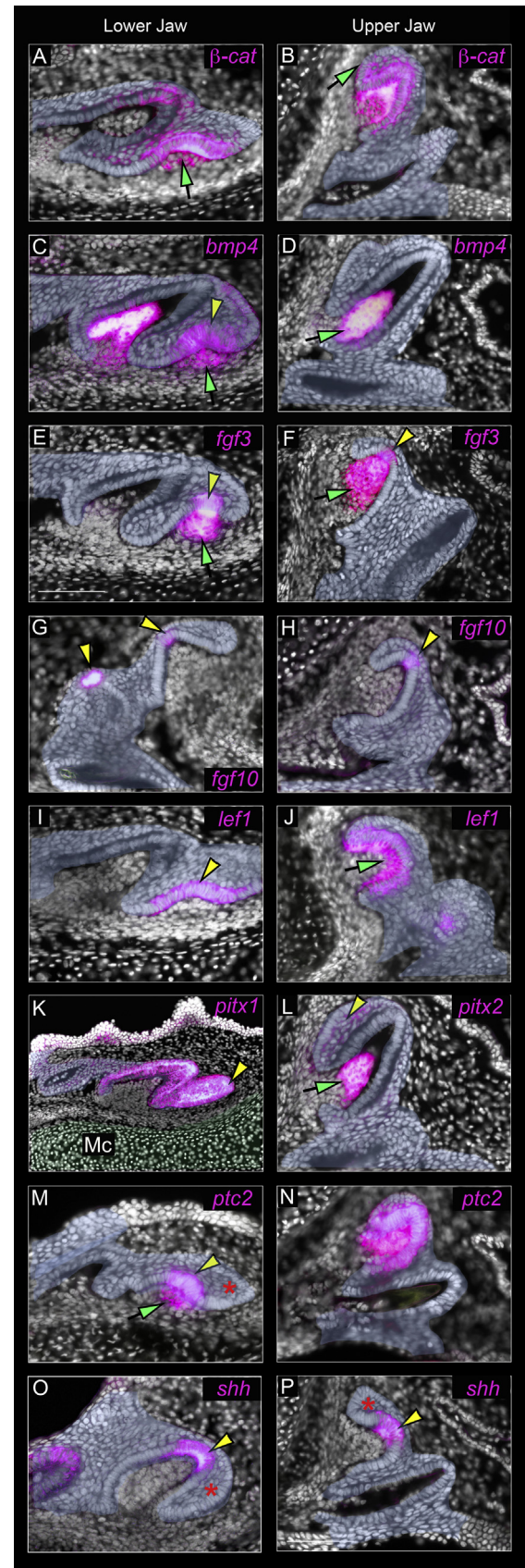
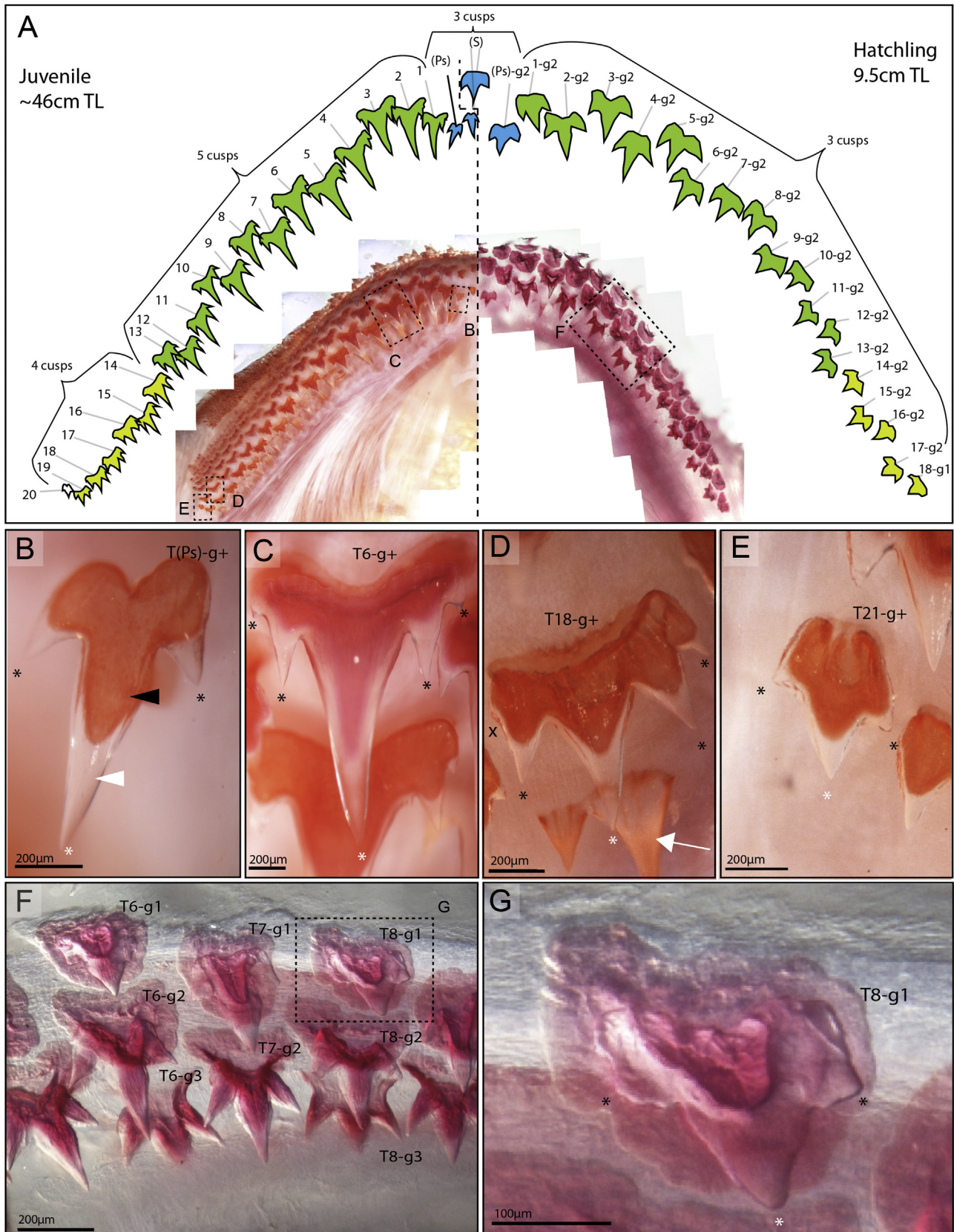


Fig. 10. Genetic conservation during continuous tooth regeneration from the deep dental lamina in sharks. Stages represent the two-three tooth generation phase of development. A, C, E, G, I, K, M, O, show images of the lower jaw dentition, while B, D, F, H, J, L, N, P, show images of the upper jaw dentition in *S. canicula*. False colour (magenta) *in situ* hybridization analyses from DIG-BM purple colour reaction; white cells are DAPI labelled, and the blue (false) colour highlights the definition of the dental lamina epithelium. A and B, β -catenin expression in epithelial and condensing mesenchymal cells (green arrow) of the successional lamina, and in early cap-stage replacement teeth. B, β -catenin is expressed in the maturing replacement tooth in cells of the inner dental epithelium and the dental papillary mesenchyme. C and D, *bmp4* expression restricted to the dental papilla at mature stages of tooth morphogenesis (green arrow in D); at stages of replacement initiation *bmp4* is expressed in the apical cap epithelium (IDE; yellow arrowhead in C), and in the directly underlying condensing mesenchymal cells of the early papilla (green arrow in C). E and F, *fgf3* expression is restricted to mesenchymal cells (green arrows) in the early cap-stage replacement teeth at the end of the dental lamina (successional lamina), at this time *fgf3* is also expressed in the presumptive primary EK-like apical cells in a small subset of the inner dental epithelium (IDE; yellow arrowheads). G and H, *fgf10* has a similar epithelial gene expression pattern to *fgf3*, and is specific to the few cells of the EK-like cells of the IDE (yellow arrowheads), at several stages of early replacement tooth formation. I and J, *left1* expression is present in both epithelial and mesenchymal cells of the developing replacement teeth; stronger *left1* expression is observed in epithelial cells at the onset of tooth replacement at the successional lamina (newest teeth), and more diffuse expression in both the cap-stage IDE and underlying mesenchymal cells of the papilla. K, *pitx1* expression in the lower jaw dental lamina and replacement tooth epithelium, and the successional lamina (yellow arrowhead). L, *pitx2* expression in the upper jaw replacement teeth, in the dental papilla during stages of morphogenesis (green arrow), and in cells of the successional lamina epithelium (yellow arrowhead). M and N, *ptc2* is expressed in both mesenchymal (green arrow) and epithelial cells (yellow arrowhead) of the developing replacement teeth. Expression shows polarity in early cap-stage teeth (M); however, like *shh* (O, P) expression of *ptc2* is not present at the limit of the successional lamina (red asterisk in M, O and P), and therefore is not associated with replacement tooth initiation. O and P, *shh* expression is observed in the IDE, associated with the primary EK-like signalling centre (yellow arrowheads). *shh* is associated with tooth morphogenesis and not replacement tooth initiation (red asterisk). Mc (green colour), Meckel's cartilage.

going mineralization, while the youngest tooth generations are in the morphogenesis, or bud stage. The entire first generation tooth row erupts prior to the hatching phase of development, thus hatchling sharks emerge fully prepared to feed (Figs. 1 and 11). It

has previously been shown that in some vertebrates, including teleost fishes (Fraser et al., 2012), the process of rudimentary tooth formation ahead of the fully regenerative and functional DL might be conserved among some polyphyodont vertebrates, i.e.



actinopteryians (Fraser et al., 2006a). Interestingly, a rudimentary placeholder generation of teeth is not observed in mammals, which generally form a maximum of two (diphyodont) tooth generations (Jernvall and Thesleff, 2012). Although, it has been shown that pre-lacteal teeth in incisor regions can form in the mouse (Hovorakova et al., 2011), and this could provide contrary evidence of a mammalian rudimentary generation.

The first-generation teeth in the shark emerge concurrently with the formation of the DL as thickenings of the OB that contort to become shallow cap-shaped tooth buds at the jaw margin (Figs. 2 and 9). However, the first generation teeth in sharks are superficial and rudimentary, forming basic cusps of dentine and enameloid (Figs. 1, 2 and 11). Mineralised tooth cusps overlay large and initially amorphous (osteodentin) root-bases that are ankylosed to connective tissues surrounding the jaw cartilages (Reif, 1980; Smith et al., 2009a; Figs. 1, 2 and 11). The first teeth to develop in the catshark (*Scyliorhinus* spp.) are irregular with variable cusp numbers (1–3, depending on the jaw position of the tooth; Figs. 1E–H and 11) and sizes compared to the subsequent generations.

3.4. The continuously regenerative dentition of the catshark develops from a permanent, deep dental lamina

Probably the most distinctive character of the shark (and ray) dentition is the propensity for continuous regeneration of multiple formidable rows of teeth, within which multiple generations of teeth may be simultaneously functional (Figs. 1 and 11). The catshark arrives at this impressive dental phenotype via the continuous redeployment of key tooth developmental genes in both the epithelial cells of the permanent DL, and underlying odontogenic mesenchyme (Fig. 10), which must each retain a degree of pluripotency (stemness) throughout the adult life of the shark. The permanent DL of the catshark is a continuous jaw-length epithelial structure nestled within the recesses of the jaw cartilages (Fig. 2). Within this DL new teeth are initiated and undergo morphogenesis before finally becoming functional upon eruption as the whole 'conveyor-belt' of teeth at different developmental stages moves labially towards the edge of the jaws (Figs. 9–11). The DL forms through proliferative growth (Figs. 6 and 7) coincident with the initiation of the first generation tooth sites and sets up the precisely timed development of subsequent generations in distinct tooth families in both the upper and lower jaws (Fig. 8). Following first generation initiation, the controlled outgrowth of the shark DL retains its general shape and cellular boundary integrity with a well-defined basement membrane surrounding the successional lamina (Figs. 9 and 10). The successional lamina is the distal extent

(free-end) of the DL (Fig. 2) and is the site of new replacement tooth initiation (Fig. 10).

Genes associated with the Bmp, Fgf, Wnt/B-catenin, and Hedgehog signalling families are expressed within cells related to all stages of tooth regeneration in the shark dentition, from the early DL (Fig. 5) to the regenerative successional lamina (Figs. 10 and 12). Within the successional lamina the inner cells emerge to form the tooth cap; the outer epithelial cells that form the lingual extent of the DL surround a core intermediate lamina cell set that expresses a number of markers including *pitx1* and *pitx2* (Fig. 10). This expression somewhat overlaps with expression domains of β -catenin, (Fig. 10A and B) and it has been hypothesized (Smith et al., 2009b; Tucker and Fraser, 2014) that one of two proposed pockets of epithelial stem/progenitor cells reside in this intermediate successional lamina region (Figs. 9 and 10, asterisk in Fig. 10M, O, and P). The second proposed region of epithelial stem cell maintenance necessary for tooth regeneration may reside in more superficial cells at the junction between oral epithelial cells that are on the cusp of becoming lamina epithelium and oral taste territories (Figs. 2, 5 and 9F, asterisk). These potential sites of epithelial stem cell activity are in accordance with other polyphyodont vertebrates e.g. reptiles (Gaete and Tucker, 2013; Handrigan et al., 2010; Handrigan and Richman, 2010b; Wu et al., 2013). The relationship between these sites and the expression of stem cell markers are currently being investigated (Martin and Fraser, unpublished). At the free end of the DL (successional lamina) a number of genes are expressed to induce tooth replacement initiation including β -catenin, *lef1*, and *pitx1* (Figs. 10 and 12). Interestingly, *shh*, known to be expressed in sites marking initiation of the first tooth generation, is not expressed in initiatory sites of tooth replacement emergence (Fig. 10O and P, asterisk). This is confirmed in the polyphyodont snake (Handrigan and Richman, 2010a) and cichlid (Fraser et al., 2013).

The ability of elasmobranchs to produce this highly productive and deep lying DL has been key to their success as a major vertebrate lineage. Maintenance of this structure is absolutely necessary for the continued regeneration of teeth. It is plausible that mesenchymal factors are necessary for the maintenance and reciprocal signalling required for growth of this dynamic lamina. *pax9* (Fig. 9I and J) and *pitx2* (Fig. 9M and N) are two mesenchymal markers in the shark that appear to surround the successional lamina during at least the initiation of the second tooth generation (Fig. 9); it will be important to focus research efforts on the role of mesenchymal factors that either help maintain DL integrity or maintain a population of stem-like cells necessary for tooth regeneration (Kaukua et al., 2014).

Fig. 11. Generational heterodonty and changes in tooth morphology during development and growth in *Scyliorhinus canicula*. A, Schematic illustration of the *S. canicula* lower jaw dental formula in hatchling (right) and juvenile/adult (left) animals based on cleared and alizarin red stained specimens. Tooth morphology varies both with jaw position and with developmental timing in *S. canicula*. B, In the adult dentition, both symphysial (S) and parasymphysial (Ps) midline tooth positions invariably exhibit a tricuspid phenotype as exemplified by an advanced cusp mineralization stage parasymphysial T(Ps) replacement tooth of unknown generation (g+) in with cusp positions demarcated by asterisks (*), in white for main cusp, and in black for accessory cusps. In advanced cusp mineralization stage replacement teeth, a clear boundary can be seen between the hyper-dense enameloid caps (transparent, white arrowhead) and alizarin-red positive dentine (red, black arrowhead). Within the lateral tooth fields, tooth morphology varies with jaw position with the largest teeth generally more proximate to the midline, and reducing in size towards the jaw margins. C, Cusp number and configuration also varies with jaw position with the teeth closer to the midline exhibiting a pentacuspid morphology with a single prominent main cusp (white asterisk) and 2 pairs of accessory cusps (black asterisks) characterizing tooth families 1–13, as exemplified by the high magnification image of unknown replacement generation of tooth family T6. D, More lateral tooth positions exhibit progressively reduced prominence of the central cusp (white asterisk) and increased prominence of 3 accessory cusps (black asterisks) coupled with the loss of the final accessory cusp most distal from the midline (black 'x') and exhibit a quatercuspid morphology, as exemplified by tooth T18-g+ and characteristic of tooth families 14–19 at this stage. Younger replacement generations at early cusp mineralization stage exhibit light alizarin red staining of the enameloid in the emerging cusps (white arrow), prior to dentine mineralization and enameloid hypermineralization, which will eventually exclude alizarin red. E, As the individual grows, additional tooth families are added to the distal jaw margins (T19, T20) beyond the complement present in hatchling-stage animals (A, right hand side), and the most recent tooth family added (T20) exhibits a tricuspid morphology with one central cusp (white asterisk) and two accessory cusps (black asterisks) reminiscent of the symphysial and parasymphysial teeth, and is significantly smaller than adjacent lateral tooth families. F, In hatchling stage animals, which possess 18 lateral tooth families tooth development is staggered with even-numbered tooth families showing up to 3 mineralization-stage generations, while odd-numbered tooth families exhibit only up to 2 mineralization stage generations. Tooth families more distal from the midline have initiated progressively fewer replacement generations and the very last tooth family to initiate (T18) only comprises of 1 generation exhibiting mineralization at hatching. G, Cusp morphology of the first generation (g1) dentition in *S. canicula* is variable and rudimentary compared with subsequent replacement generations usually with only a single definitive central cusp (white asterisk) and 1 or 2 rounded, irregularly shaped accessory cusps (black asterisks), exemplified by T8-g1.

3.5. Signalling centres for tooth cusp morphogenesis are conserved between sharks and bony vertebrates

The teeth of sharks are highly diverse in both shape and cusp ornament, from blade-like cutting teeth, with or without serrations, to broad, pavement-like crushing teeth (Cappetta, 2012). The teeth of *Scyliorhinus* spp. are sharp, elongated, non-serrated units with a larger main (central) cusp and up to two pairs of accessory cusps (Ellis and Shackley, 1995; Figs. 1 and 11). In mammals, the enamel knot (EK) is an important signalling centre directing the formation of definitive cusps (Jernvall et al., 1994; Jernvall and Thesleff, 2012; Vahtokari et al., 1996). During morphogenetic stages of catshark tooth development, we observe a signalling centre at the apex of the emerging central cusp, which bears both positional and genetic similarities to the mammalian primary EK, and is therefore likely to be homologous (Figs. 6, 7 and 9). Although, histologically this apical epithelial structure in the shark is not 'knot-shaped' and thickened as observed in mammalian EKs, it does show a distinct clustered, columnar set of inner dental epithelial cells that express a set of markers that include *bmp4*, *fgf3*, *-10*, *midkine*, *ptc2* and *shh* (Figs. 9 and 10). With both positional and genetic clues to the nature of this signalling centre, we also observe the non-dividing characteristic of the shark primary EK, in both first generation and in subsequent generations of tooth replacement. PCNA assays offer evidence that the shark EK is a non-dividing population of epithelial cells with an obvious lack of PCNA in the apical cells (Figs. 6F and 7F–H). Thus, the cessation of mitotic activity in these restricted regions of the developing dental epithelium might infer control of shark tooth shape, as is the role of the likely homologous signalling centres in other vertebrates, including mammals (Jernvall et al., 1994; Vahtokari et al., 1996). In mammals, the distinct and limited cluster of non-dividing apical epithelial cells in the cap-stage tooth making up the EK participates in reciprocal molecular interactions with the underlying mesenchymal cells during cap-bell stage tooth morphogenesis through the expression of secreted signalling factors (Jernvall and Thesleff, 2000). In the catshark, as morphogenesis begins at the early cap-stage of tooth development, the apical cells of the inner dental epithelium (Figs. 6 and 7), which mark the tip of the primary cusps, express several of the same secreted signalling factors as the EK of mice including *fgf3*, *fgf10*, *bmp4*, and *shh* (Fig. 10). This subset of secreted signalling factors are also expressed in epithelial cells of mammalian EK signalling centres (Jernvall and Thesleff, 2000), demonstrating the remarkable conservation of key genetic components of tooth signalling centres between sharks and mammals. The first indication of primary EK-like epithelial cells in *Scyliorhinus* occurs during the formation of the first generation teeth, at the cap stage of tooth development (Figs. 6 and 7). In

terms of expression of knot markers, there is little change in expression from the rudimentary first generation teeth to the second and further generations of more adult-like teeth (Figs. 9 and 10). *fgf3* is expressed both in the EK-like cells of the epithelium and the papillary mesenchymal cells directly underlying the EK (Fig. 9E, F and 10E, F). *fgf10* is only expressed in a small number of cells and limited to the EK-like apical epithelial cells of shark teeth at most stages of tooth morphogenesis (Fig. 10G, H and 12). *bmp4* is

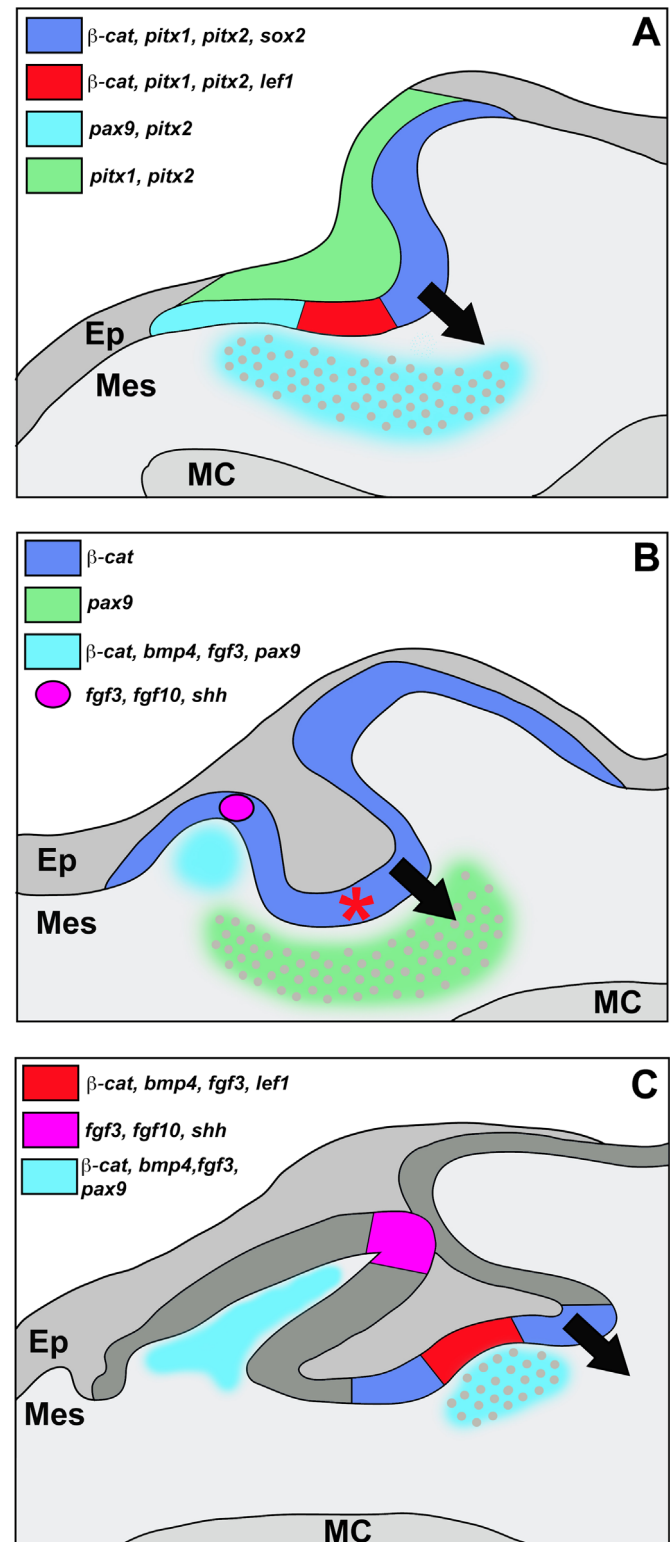


Fig. 12. A core dental gene network for tooth initiation, and regeneration in sharks. Schematic diagram summarizing the gene expression profiles during shark odontogenesis. A, the thickened dental epithelium starts the development and growth (black arrow) of the dental lamina toward the underlying cartilage (MC). The deep epithelial site at which the first tooth will emerge (red) expresses *lef1*, and not *sox2* (dark blue). The underlying mesenchyme condenses and marks the region (light blue, dotted) where the dental lamina will invaginate. B, the first tooth begins its development and forms a contorted, superficial cap-shaped structure with papillary mesenchyme and an enamel knot-like unit (pink circle), expressing the characteristic markers *fgf3*, *fgf10* and *shh*. During first tooth formation the dental lamina continues its growth and proliferation (black arrow) into the underlying condensing mesenchyme (dotted green) that surrounds the future successional lamina (red asterisk). C, next (second) generation teeth begin to emerge from the successional lamina, as a cap-shaped unit with inner dental epithelium (red) expressing β -catenin, *bmp4*, *fgf3*, and *lef1*. The outer dental epithelium of the successional lamina (dark blue) will continue to proliferate to form future tooth generations (black arrow). The maturing first tooth retains an apical unit similar to the mammalian enamel knot (pink). Ep, epithelium; Mes, mesenchyme; MC, Meckel's cartilage.

Table 1

Core gene expression among major clades of toothed vertebrate, with the addition of the shark. Comparative expression of core genes in the dental epithelium (E, pink) and dental mesenchyme (M, blue) during tooth development and replacement (in the Shark, Bony Fish, Reptile, and Mammal (presence of expression denoted by +, and absence of expression denoted by -). Bony Fish: (1, *Metriaclichia zebra*; Fraser et al., 2013; 2, Fraser et al., 2009); Reptile (3, *Python regius*; Handrigan and Richman, 2010b); Reptile (various) and Mammal (various; 4, Juuri et al. 2013); Reptile (5, (*A), *Crocodylus siamensis*; pax9 expressed in oral mesenchyme of early forming tooth regions (Tokita et al., 2012), although expression in *A. mississippiensis* at later stages is shown to be present in the dental lamina (epithelium) however, this is not clear; Weeks et al., 2013); Mammal: (6, *Mustela putorius furo*; Jussila et al., 2014); Mammal (7, *Mus musculus*; St Amand et al., 2000); Mammal (8, (*B), *Monodelphis domestica*; fgf10 is predominantly expressed in dental mesenchyme (e.g. in *Mus musculus*, 9, Kettunen et al., 2000), however, some expression data suggests fgf10 is also associated with primary enamel knot epithelium in e.g. *M. domestica*; Moustakas et al., 2011). (? , question mark denotes unknown expression patterns during tooth development and replacement.

	Shark		Bony Fish ^{1,2}		Reptile ³⁻⁵		Mammal ⁶⁻⁹	
	E	M	E	M	E	M	E	M
<i>β-cat</i>	+	+	+	+	+	+	+	+
<i>bmp4</i>	+	+	+	+	+	+	+	+
<i>fgf3</i>	+	-	+	-	?	?	+	+
<i>fgf10</i>	+	+	+	+	+	+	+	(-)* ^B
<i>lef1</i>	+	+	+	+	+	+	+	+
<i>pitx2</i>	+	+	+	-	+	-	+	-
<i>pitx1</i>	+	-	?	?	?	?	+	-
<i>pax9</i>	-	+	-	+	?	?* ^A	-	+
<i>shh</i>	+	-	+	-	+	-	+	-
<i>sox2</i>	+	-	+	-	+	-	+	-

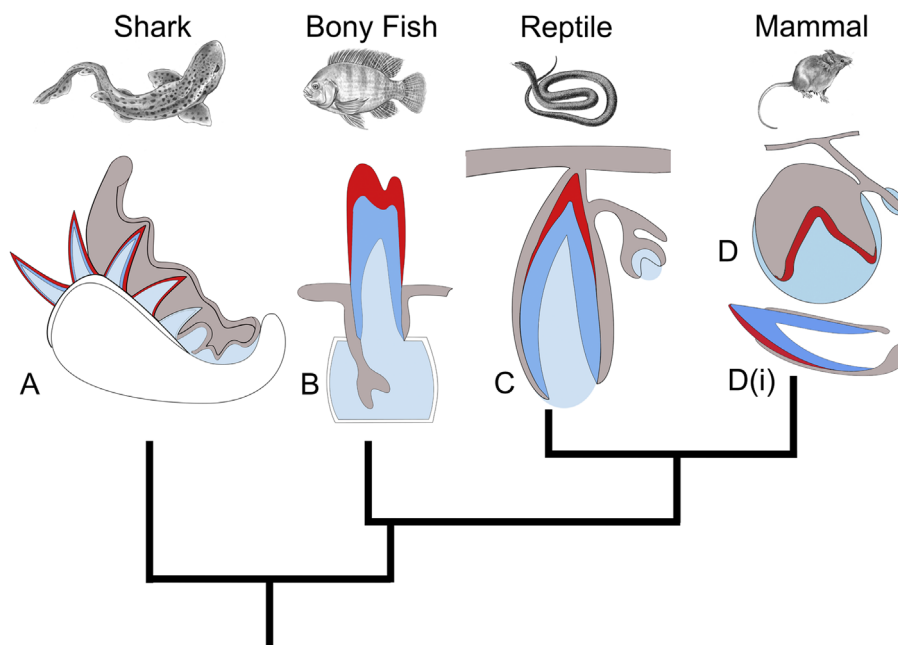


Fig. 13. Comparative phylogeny of tooth replacement modes in toothed vertebrate clades. A. the shark dentition (elasmobranchs) continuously regenerates throughout life (polyphyodont) with a many-for-one mechanism (many teeth made in advance of function for each functional tooth position in the jaw). B. Bony fishes offer a diverse range of tooth replacement mechanisms, with continuous replacement with a one-for-one system (e.g. cichlid fishes, in B (Fraser et al., 2013); one replacement tooth made in advance of function for each functional position), although a many-for-one system is observed in many bony fish species. C. Reptiles show a range of diverse mechanisms for tooth replacement with a continuous polyphyodont system in some species e.g. the snake, in C (with some species showing a more restricted dentition without replacement (monophyodont; e.g. chameleon; Buchtová et al. 2013). D. The mammalian dentition also offers a range of diverse replacement or renewal phenotypes from monophyodont mammals e.g. mice, where the only regenerative potential is observed in the continuously growing incisor (Di), and molars are never replaced. In most mammals the dentition is diphyodont with two tooth generations e.g. humans (D). A phylogenetic reduction of tooth generations are generally observed toward more higher vertebrates i.e. mammals.

expressed in both the early mesenchymal papilla and the inner dental epithelial cells of the early cap-stage tooth in the first and subsequent generations of teeth in *Scyliorhinus* (Fig. 9C, D and 10C, D). *bmp4* expression in these two adjacent cell compartments is

similar to known expression in the mammalian molar during morphogenesis where these tissues interact (Jernvall et al., 1998), and here shark teeth show equivalent co-expression of *fgf3*, *β-catenin* and the canonical Wnt pathway read-out transcription

factor, *lef1* (Figs. 9 and 10). This expression pattern, both in the apical epithelial cells of the presumptive primary EK-like structure in the shark, and the directly underlying dental papillary cells, is highly similar to the molar tooth of the mouse (Jernvall et al., 1994; Vaahrokari et al., 1996). The genetic and morphological conservation of both initiation and growth of the tooth is relatively unchanged in vertebrates, and perhaps surprisingly the signalling centre that directs cusp morphogenesis has been retained throughout vertebrate evolution. This highlights the stable developmental genetic cascade and cellular response in this process. An inner enamel epithelium-derived signalling centre therefore acts as an organizational centre in both bony fishes (at least the primary EK) (Jernvall and Thesleff, 2000) and cartilaginous fishes and has likely directed tooth shape and cusp morphogenesis since the first vertebrate tooth.

4. Discussion

4.1. A tooth by any other name... Highly diverse dentitions utilize the same developmental toolkit

Gene expression during stages of tooth development in the shark highlights the highly conserved genetic mechanism that forms the basis to the core 'odontode' unit: tooth-like structures including teeth, skin denticles and other skin ornaments composed of dentine and enamel-like tissues (Debiais-Thibaud et al., 2011; Donoghue, 2002; Fraser et al., 2010). Vast diversity and complexity can arise from these forms. Core markers of tooth competence and development are present in all dentitions from sharks to mammals, a 450 million year conservation of a core dental network. Even the earliest core components of the dental developmental programme, albeit latent, exist in toothless vertebrates e.g. Aves (Chen et al., 2000), highlighting the early competence of an odontogenic pre-pattern in, at least, the oral epithelium prior to subsequent loss of tooth initiatory competence thought to be associated with loss of reciprocal ectomesenchymal signalling (Chen et al., 2000; Mitsiadis et al., 2006, 2003). This further suggests that an early core dental network exists in most vertebrates, and likely all vertebrate clades. A derived loss of either all teeth or just the tooth regeneration programme can occur via developmental restriction through loss of compartmental signalling (developmental tinkering). Similarly, the enhancement of the dentition to form a continuous supply of teeth without restriction is governed by the maintenance of these factors capable of tooth competence and concurrent modification of the timing, shape and regenerative potential. Both restriction and enhancement of dentition and replacement is a product of both gene network tinkering and stability of the core genetic components. Sharks (and elasmobranchs more generally, including the rays) are a particularly important set of models for the complete understanding of the tooth development and dental regenerative programmes.

Overall, this examination of tooth initiation, development and regeneration in the shark offers insights into the basal characters of the vertebrate dentition, and importantly highlights the high level of conservation of these core dental genes in vertebrate tooth development (Table 1; Fig. 13). These data suggest that the broad spectrum of vertebrate odontogenesis must retain this core set of genes, regardless of type and phenotypic restriction of the specific system. Some elements that show significant differences between sharks and all other vertebrates could emphasize more basal components of the earliest vertebrate dentition. In the developing shark *pitx2* has a unique expression pattern, present predominantly in dental mesenchymal cells throughout odontogenesis (in addition to dental epithelial expression; Figs. 3–5, 9 and 10; Table 1). This is an uncommon expression pattern among

vertebrates and could suggest an ancestral character and a potential mesenchymal role for *pitx2* in the formation of the shark dentition, and likely the earliest dentitions of basal vertebrates. In other vertebrates that have been documented (teleost fishes, reptiles and mammals; Table 1) *Pitx2* is exclusively expressed in dental epithelial cells, a pattern not so restricted in the shark. It is intriguing that sharks (i.e. *Scyliorhinus*) therefore have retained (or acquired) a mesenchymal expression pattern for *pitx2* in early tooth site regionalisation (prior to tooth initiation; Figs. 4D–F and 5G, H) through morphogenesis and even during dental regeneration (Fig. 9M, N and 10L), which could be related to an ancestral mesenchymal role for *Pitx* genes, lost in most vertebrates. Therefore, these data could suggest a potential mesenchymal role for *pitx2* as a distinctive character for basal vertebrate dentitions.

It is thought that sharks retain a set of basal vertebrate dental characters and that at the dawn of toothed, jawed vertebrates, a continuous production of teeth was commonplace. We suggest that for this basal dental system of continuous tooth regeneration, a dental lamina was and is absolutely necessary, and equivalent to that seen in the extant shark (Figs. 10–12). To maintain a continuous tooth regenerative programme or even a limited but multi-generational dentition, an extension of the dental epithelium (dental lamina) must have the ability to continue proliferation from a stock of stem-like progenitor cells to form new tooth generations (Tucker and Fraser, 2014). To function, this lamina must be capable of receiving the inductive signals necessary to maintain the reciprocal signalling with the surrounding mesenchyme. Additionally, these competent epithelial cells should have some ability to respond to potential signals originating from mesenchymal stem-like cells that likely persist in some form of latent state (Kaukua et al., 2014; Zhao et al., 2014).

4.2. Formation of the odontogenic band—a cellular pre-pattern for tooth initiation

The early epithelial gene calibration and the onset of tooth competent cells demarcating the odontogenic band (OB) is a fundamental character of the developing vertebrate jaw. We therefore suggest that for teeth to appear from a seemingly homogeneous oral cell condensation in vertebrates, an OB must be a prerequisite. The process of tooth development is evolutionarily stable, a conserved process that can undergo minor shifts in the deployment of genes during morphogenesis that can alter dental morphology; however the overall core dental network remains set, governed by stable vertebrate pathways (Hariharan et al., 2015). The stability of these dental pathways unites diverse dentitions from the emergence of tooth competent cells within the OB through tooth development and replacement. If tinkering with the core genetic pathways for tooth development alters shape and creates diverse phenotypes, then the same conserved programme must exist for tooth replacement, where genetic tinkering (Jacob, 1977; Lieberman and Hall, 2007) could facilitate a reduction or expansion of the capacity for tooth replacement (Fraser et al., 2013; Jernvall and Thesleff, 2012). This may suggest that slight genetic alterations could be the difference between a limited, mammalian replacement supply and an unlimited dentition as presented here in the shark. This is a promising opportunity for the future of tooth regenerative biology, and sharks could play an important role in the discovery of new methods and key markers for continuous tooth regeneration.

4.3. Development of a dental lamina, not necessary for odontogenesis but essential for dental regeneration

The first generation dentition in sharks is superficial and rudimentary, and this process is conserved among most vertebrates

except mammals. First rudiments develop superficially and without the need for a 'dental lamina' proper. Interestingly, the dental lamina seems to be a highly dynamic structure that is required for the developments of multi-generation dentitions (Smith et al., 2009b). However, among the great diversity of fishes, some species have retained the ability to develop continuously replacing teeth without a 'standard' dental lamina, e.g. salmonids; instead their repeated dentition arises from epithelial cells associated with the development of predecessor teeth (Fraser et al., 2006a; Huyseune and Witten, 2008). This could be characterized as a basic lamina, from cells lying deep to the predecessor tooth for the continued production of tooth replacements in a one-for-one manner. A 'one-for-one' tooth replacement state could be considered atypical of polyphyodont vertebrates and could relate to why some teleosts such as the salmonids (Fraser et al., 2006a; Huyseune and Witten, 2008) are able to replace their teeth without the need for a true dental lamina, whereas, species with a 'many-for-one' replacement system must do so with a dental lamina. Sharks and their relatives (including the rays), however, continuously regenerate their dentition with a 'many-for-one system' (many teeth within a single family/file for a single functional position (Figs. 1, 11 and 13; Tucker and Fraser, 2014), where individual functional teeth at the jaw edge are followed by many lingual tooth replacements that develop ahead of function within the deep epithelial dental lamina. The 'many-for-one' polyphyodont dental system is observed in many vertebrates outside the elasmobranchs, including reptiles (Fig. 13; Gaete and Tucker, 2013; Handrigan et al., 2010; Handrigan and Richman, 2010b).

The dental lamina is an essential requirement for multiple tooth generations in vertebrates. The dental lamina as an extension of the dental epithelium, associated with the predecessor tooth, is a vital vertebrate character for polyphyodonty (Fraser et al., 2013; Gaete and Tucker, 2013; Handrigan et al., 2010; Jernvall and Thesleff, 2012) and enables the variable regenerative capacity for new teeth. The degradation of the dental lamina in some vertebrates after the first or second-generation dentition coincides with limited potential for regeneration, e.g. in mammals including the mouse (Jarvinen et al., 2006), ferret (Juuri et al., 2013), and human (Volponi et al., 2010; Fig. 13). Here we show the shark as an extreme example of dental lamina diversity with an expanded dental lamina housing several generations of teeth in advance of function, in association with the chondrichthyan character of unique cartilaginous jaws (Fig. 13). Without the dental lamina tooth regeneration would likely be severely disrupted, and we predict only first generation teeth would form, at least in the shark. This hypothesis is eminently testable and future experiments on elasmobranch models will prove the influence of the dental lamina as essential for continuous and rapid tooth regeneration. It is intriguing that higher vertebrates that have a greatly reduced dentition, in terms of replacement, also fail to retain the dental lamina. Typically replacement fails to occur after the break down or loss of the dental lamina in mammals (Buchtová et al., 2012).

The shark dental lamina importantly houses epithelial cells that likely have stem-like properties (Martin and Fraser, unpublished), as reported in several vertebrates with a polyphyodont dentition (Gaete and Tucker, 2013; Handrigan et al., 2010; Juuri et al., 2013). This is a crucial role for the dental lamina and one that suggests a dental lamina is essential for the continued production of tooth replacements. The exact location of putative stem cells within this distinctive regenerative character in the shark is yet to be confirmed (Martin and Fraser, unpublished), however it has been hypothesized that stem/progenitor cells could exist in one of two locations of the dental lamina (Smith et al., 2009a): either the distal successional lamina (Figs. 10 and 12) in the intermediate cells of the middle epithelium (Huyseune and Thesleff, 2004) or within a pocket of superficial epithelia linking the dental lamina to

the taste bud-dense oral epithelium (Figs. 2 and 8).

The occurrence of supernumerary teeth in humans and other mammals suggests some aberrant or anomalous genetic mechanisms that can lead to a shift, albeit, rare, in the development of the human/mammalian dentition (Wang and Fan, 2011). This hints at a possible dormant availability of potential material for stimulated extension of the limited dentition. A number of reports offer genetic association e.g. Wnt/ β -catenin (Jarvinen et al. 2006; Wang et al. 2009), Lef1 (Zhou et al., 1995) and Runx2 (Wang and Fan, 2011), and genes associated with these perturbations are expressed in the shark dentition (shark runx2 data not shown) at sites of replacement potential. With this model system it will be useful to design manipulation experiments based on evidence gathered from expression of genes involved specifically in the expansion, maintenance or elaboration of the dental lamina to investigate further in mammals.

4.4. The enamel knot-like signalling centre – a vertebrate innovation?

Based on these gene expression (Figs. 9 and 10) and immunohistochemical data (Figs. 6 and 7), we suggest that all teeth share a mechanism for tooth shape that is governed by enamel knot-like signalling centres (Jernvall et al., 1994). However, the defining characteristics of the EK apoptotic mechanism in the shark dentition have yet to be confirmed. We suggest that the first teeth to emerge during the evolution of vertebrates utilized a common set of genes for tooth morphogenesis and to establish cusp shape, whether forming simple unicuspid teeth or multi- cuspid forms in sharks (Figs. 1 and 11) or more complex dental phenotypes for example molar teeth of mammals (Jernvall and Thesleff, 2000, 2012; Thesleff and Sharpe, 1997).

Interestingly, cusp morphogenesis in some vertebrates (e.g. squamates (Handrigan and Richman, 2011) and the alligator (Weeks et al., 2013)) show contrasting evidence for cusp signalling centres throughout vertebrates. These reptiles show expression of *shh* and *fgf4* in all inner dental epithelial cells rather than in a defined subset of apical cells suggestive of an EK. Thus it has been suggested that the EK is a possible mammalian innovation (Weeks et al., 2013). However, we observe a structure similar to the primary EK in the shark (Figs. 9, 10 and 12), and others have noted this structure in several species of 'lower' vertebrates, which have unicuspid and multicuspid dentitions, even during the first and second-generation teeth (Fraser et al., 2008, 2013). Therefore we suggest that at least the primary EK is a highly conserved signalling centre for tooth morphogenesis and shape diversity, and is a vertebrate innovation. However, if key markers and cellular integrity of a 'standard' primary EK are not present in apical inner dental epithelial cells (as recently purported in the Alligator and other reptiles) then it is likely that the EK can be modified or lost. Potential loss of the EK (either the primary EK or subsequent EKs) as a derived character highlights the diversity of odontogenesis. Those vertebrates with a simple dentition (e.g. unicuspid homodonts) could exhibit simplified tooth morphogenesis secondary to EK loss; further investigation will certainly uncover modifications and enhancements of EK-like cells in more derived groups of vertebrates.

Due to the lack of sequence data from shark libraries, orthologs of mammalian genes e.g. *fgf4* and *fgf9* were not cloned for gene expression analysis. However, the discrepancy in gene expression observed between the shark and mouse tooth development (Table 1) could reflect shifts in differential gene expression associated with EK-like signalling, for example *fgf10* expression in the EK-like cells of the shark (Fig. 10). Although, *Fgf10* is expressed in the presumptive dental epithelium of mouse molar regions at E10 (Kettunen et al., 2000), it becomes down-regulated in the

epithelium and only expressed in the dental mesenchyme in developing teeth from mouse E11. However, it has been reported (Moustakas et al., 2011) that *Fgf10* is expressed in the primary enamel knot in developing opossum (*Monodelphis domestica*) teeth, in addition to expression in the dental mesenchyme (equivalent to the mesenchymal expression observed in mice; Kettunen et al., 2000). The presence of *Fgf10* in the primary EKs of opossum teeth has been suggested to promote rapid growth of the epithelium providing the characteristic pointed crowns in *M. domestica* (Moustakas et al., 2011). This observation fits with both the expression pattern and tooth cusp characteristics of *S. canicula* with rapid tooth growth and sharp blade-like cusps. It will be intriguing to investigate the cellular dynamics and genetic mechanisms of tooth development and cusp morphogenesis in flat-cusp teeth in alternative elasmobranchs, such as the ray (*Raja spp.*; Underwood et al., 2015).

Shifts in gene expression might be expected given the vast phylogenetic distance between sharks and mammals, as already documented in this analysis for other genes, e.g. mesenchymal expression of *pitx2* (Figs. 7G, H, and 9M, N). We therefore hypothesize that EK-like signalling centres themselves are highly conserved among vertebrates, with similar cellular characteristics and signalling pathways in operation (e.g. Fgfs), even if only some elements of the specific genetic repertoire are equivalent. Here we have documented genetic and cellular conservation in the presumptive shark primary EK-like structure, however, complex tooth shapes arise from the formation of secondary and subsequent EK-like structures, at least in the mouse molar (Kettunen et al., 2000).

These data further emphasize the diversity of tooth shape and the mechanisms responsible for shape shifting among vertebrate clades (Jernvall and Thesleff, 2012). Genetic factors involved in tooth shape are common elements of the tooth developmental cascade and have also been shown to be associated with the cooption of signals for tooth regeneration in a range of vertebrates (Fraser et al., 2013; Jernvall and Thesleff, 2012). Sharks show a huge diversity of tooth shapes from flattened molariform cusps to sharp, blade-like teeth with or without accessory cusps and additional serrations (Cappetta, 2012). The first teeth, however, share basic morphological characters with subsequent teeth within elasmobranchs; in *Scyliorhinus spp.* the initial dentition is multicuspate and their form is comparable to teeth of adults. This is similar to lamniform sharks (Shimada, 2002), in which these first teeth, albeit peg-like and rudimentary, may be functional for a considerable period prior to birth and used for intrauterine oophagy and even embryophagy ('intrauterine cannibalism') (Gilmore, 1993).

The teeth of *Scyliorhinus* develop and pattern cusps in a similar manner to other multicuspate species, with the central cusp forming ahead of the accessory cusps, followed by the right lateral then left lateral cusp (Figs. 1 and 11). This observation suggests that tooth shape must follow conserved genetic mechanisms that have been retained throughout the evolution of vertebrates, and similar tooth shapes can be generated regardless of regenerative capacity. The shark (*Scyliorhinus*) shows that a standard multicuspate tooth shape can form from the emerging first generation dentition (Fig. 11). Sharks do offer an example of how continuous tooth regeneration mechanisms can permit a plastic dental system with respect to tooth shape. With rapid tooth turnover, adult *Scyliorhinus* show plasticity in sexually dimorphic tooth shape seasonally (Ellis and Shackley, 1995), allowing a shift in tooth shape via the process of regeneration to correspond to breeding seasons. This mechanism of tooth shape transition can only be observed in species with continuous tooth regeneration. Tooth shape-shifting in sharks and dental sexual dimorphism suggests that vertebrates with multiple generations can be relatively 'plastic' with respect to tooth morphogenesis (Kajiura and Tricas,

1996). Therefore rapid turnover of tooth replacements may give some vertebrates an advantage in altering mating behaviour or shifting their diet seasonally.

4.5. Collaboration between the cartilaginous jaws and tooth development facilitate the continuous conveyor belt-like dental regeneration in sharks

The characteristic shark jaw cartilages enable the regulation and precise cyclical nature of tooth regeneration in a 'conveyor-belt' system (Figs. 1, 2, and 11). Teeth in sharks are capable of physical movement (the mechanism of which is currently unknown) through the dental lamina toward functionality without the constraints of tooth to bone attachment observed in osteichthyan and sarcopterygians. Instead, the shark utilizes a set of connective tissues (Fig. 2) that envelop the oral surface of the jaw cartilages and attach to the root elements of the teeth (Figs. 1 and 2). This soft-tissue attachment allows both the functional retention of tooth units and the plasticity to facilitate movement of the teeth through the epithelial dental lamina. The teeth become secure and functional at the jaw margin and then lost when successor teeth of the same family force the predecessor over the jaw margin out of the oral cavity where they are eventually exfoliated (Figs. 1 and 2).

What makes the shark dentition unique is the ability for precisely timed continuous tooth regeneration. The dynamic and continuous dental lamina facilitates the development of multiple teeth in quick succession to form in the extended and deeply invaginated epithelia. All the teeth in the shark dentition, including all the newly forming replacement teeth are linked within the same set of dental epithelial cells of the lamina. The teeth are further linked together via connective tissues (Reif, 1980; Shellis, 1982) that support the basal tissues of the roots (bone of attachment), forming the quintessential conveyor-belt dentition that lies tightly associated with the surface of the jaw cartilages (dorso-lingual Meckel's and ventro-lingual palatoquadrate; Figs. 1 and 2). The fact that tooth regeneration in sharks does not involve bony attachment and instead utilizes the connective tissues as a foothold for the tooth roots enable a certain plasticity to the dental system allowing 'movement' of the tooth families in a labial (rostral) progression toward functionality and later physical exfoliation (or damage). The lack of restriction of teeth to fixed points on the jaw in sharks also allows the evolution of dentitions within which several teeth originating from within the same section of dental lamina are simultaneously functional, resulting in the extremely high morphological and functional diversity of shark dentitions. Furthermore, the continuous nature of the shark dental lamina and the lack of lamina restriction (a bony crypt restriction of the successional lamina is observed in other fishes with a polyphyodont dentition) (Fraser et al., 2013; Huyseune and Thesleff, 2004; Tucker and Fraser, 2014), permits the shark dentition an extremely rapid turn over of tooth units throughout life.

5. Conclusion

With these data, we integrate the genetic coordination of three main processes in the development of the characteristic shark dentition (Fig. 12); (i) tooth initiation and the formation of the distinctive dental lamina from which all future teeth are born, (ii) tooth morphogenesis and the conservation of a signalling centre (primary enamel knot) for tooth shape regulation, and (iii) the genetics of continuous tooth regeneration. This collection of dental characters in a tractable developmental genetic model, the catshark, will facilitate the expansion of experimental progress in the study of dental evolution, development and regeneration. Given

the extensive conservation at all levels of the odontogenic programme, presented here, the study of diverse organisms is therefore essential for both phylogenetic context and for further insights into the mechanisms of dental regenerative biology. These data report that the shark dentition is formed and patterned via highly conserved signals that are utilized by all toothed vertebrates; therefore sharks must be a definitive model for the complete understanding of general vertebrate tooth development as well as a unique model for the study of tooth regeneration. From the large scale patterning events to the smaller scale cellular mechanics, the shark shows great conservation of genetic expression, and therefore these early vertebrates offer an opportunity for further evolutionary and developmental knowledge on the origins of the vertebrate dentition.

The shark represents a key model for developmental diversity and the expansion of the functional dentition, with a distinct, continuously maintained dental lamina for cyclical life-long tooth regeneration. Based on the fact that genetic factors in tooth development and dental regeneration are both highly conserved and diverse among all toothed vertebrates, the information gleaned from this valuable developmental model will allow the translational progression of this field of regenerative biology. Future research will take advantage of the unique cyclic regeneration of the shark dentition to study mechanisms governing maintenance of potential mesenchymal and epithelial stem cells and replenishment of regenerative mechanisms in dentitions of limited supply.

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